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FUNDAMENTALS OF DAIRY SCIENCE

BY ASSOCIATES OF

LORE A. ROGERS

IN THE RESEARCH LABORATORIES OF THE
BUREAU OF DAIRY INDUSTRY
UNITED STATES DEPARTMENT OF AGRICULTURE

SECOND EDITION

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GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in coöperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company (Reinhold Publishing Corporation, Successors) of New York City.

The Council, acting through the Committee on National Policy of the American Chemical Society, appointed the editors, named at the close of this introduction, to have charge of securing authors, and of considering critically the manuscripts prepared. The editors of each series will endeavor to select topics which are of current interest and authors who are recognized as authorities in their respective fields. The list of monographs thus far secured appears in the publisher's own announcement elsewhere in this volume.

The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years and the fields covered by this development have been so varied that it is difficult for any individual to keep in touch with

the progress in branches of science outside his own specialty. In spite of the facilities for the examination of the literature given by Chemical Abstracts and such compendia as Beilstein's Handbuch der Organischen Chemie, Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Chemie, Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Chemie and the English and French Dictionaries of Chemistry, it often takes a great deal of time to coördinate the knowledge available upon a single topic. Consequently when men who have spent years in the study of important subjects are willing to coördinate their knowledge and present it in concise, readable form, they perform a service of the highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviews of this character that a Committee of the American Chemical Society recommended the publication of the two series of monographs under the auspices of the Society.

Two rather distinct purposes are to be served by these monographs. The first purpose, whose fulfilment will probably render to chemists in general the most important service, is to present the knowledge available upon the chosen topic in a readable form, intelligible to those whose activities may be along a wholly different line. Many chemists fail to realize how closely their investigations may be connected with other work which on the surface appears far afield from their own. These monographs will enable such men to form closer contact with the work of chemists in other lines of research. The second purpose is to promote research in the branch of science covered by the monograph, by furnishing a well digested survey of the progress already made in that field and by pointing out directions in which investigation needs to be extended. To facilitate the attainment of this purpose, it is intended to include extended references to the literature, which will enable anyone interested to follow up the subject in more detail. If the literature is so voluminous that a complete bibliography is impracticable, a critical selection will be made of those papers which are most important.

The publication of these books marks a distinct departure in the policy of the American Chemical Society inasmuch as it is a serious attempt to found an American chemical literature without primary regard to commercial considerations. The success of the venture will depend in large part upon the measure of coöperation which can be secured in the preparation of books

dealing adequately with topics of general interest; it is earnestly hoped, therefore, that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

To

LORE ALFORD ROGERS

In recognition of his quarter-century service in the advancement of knowledge, embracing important contributions in pure science as well as its applications to industry; and because he embodies in the highest degree their ideal of unselfish devotion and untiring loyalty, alike to his work and to his fellow workers—this volume is dedicated, with admiration and affection, by those who have been privileged to serve under his leadership.

Preface

This book has been written to fill a need experienced by advanced students and research workers in the field of dairy science. The effort has been made to present basic data, fundamental observations, and unbiased discussions of researches that contribute to the present status of the dairy industry. Matter has been arranged in such a way as to bring together topics considered from the same scientific angle rather than to group them under the more usual headings of specific products of dairy manufacture. Because of the fact that research has advanced farther along some lines than along others, no attempt has been made to accomplish a fine balance in relative space among topics according to their intrinsic importance. Knowledge of certain topics,—e.g., pigments, vitamins,—is advancing so rapidly that, by the time this edition is published, some parts will already be in obvious need of further alterations and additions. It is hoped that one result of the publication of this book will be to stimulate research along lines now lagging, thereby correcting somewhat the lack of balance in our knowledge of the scientific basis of the dairy industry.

All contributors to the various chapters are present or former members of the staff of the Research Laboratories of the Bureau of Dairy Industry. In the following list the present position of each former member contributing is given, if known. Except where specifically stated otherwise, contributions were revised for this second edition by the original authors.

Chapter I. Composition of Milk and Milk Products. Major differences in composition of milk by H. G. Albery, revised by P. A. Wright; Minor milk constituents by E. F. Deysher; Milk serum by Anne G. Benton, now Professor of Bacteriology in Vassar College; Enzymes by W. R. Albus, now physician in Chicago, Ill., revised by H. R. Curran; Composition of milk products by P. A. Wright.

Chapter II. Proteins of Milk. Chemistry and preparation of casein by R. W. Bell, now Chief of Division of Dairy Manufacturing Investigations and Introduction of the Bureau of Dairy Industry; Uses of casein by A. O. Dahlberg, now Chemist for the Golden State Creameries, California; Lactalbumin by P. N. Peter, deceased; Lactoglobulin by Philip Rupp, retired. Chapter revised by E. O. Whittier.

Chapter III. Milk Fat. George E. Holm and G. R. Greenbank.

Chapter IV. Pigments of Milk. L. S. Palmer, now Professor of Agricultural Biochemistry in the University of Minnesota.

Chapter V. Lactose. E. O. Whittier.

Chapter VI. Acid-Base and Oxidation-Reduction Equilibria of Milk. W. M. Clark, now Professor of Physiological Chemistry in the Johns Hopkins University Medical School. Revised by E. O. Whittier.

Chapter VII. Physical Equilibria of Milk. George E. Holm.

Chapter VIII. Coagulation of Milk. Heat coagulation by Alan Leighton, revised by B. H. Webb; Alcohol coagulation by Anne G. Benton; Rennet coagulation by L. S. Palmer; Cheese manufacture by K. J. Matheson, revised by G. P. Sanders.

Chapter IX. Freezing of Milk and Milk Products. Freezing temperatures and effects of freezing by Alan Leighton; Properties of ice cream by O. E. Williams, revised by Alan Leighton and Abraham Leviton.

Chapter X. Sources and Distribution of Bacteria Found in Milk. General sources by J. M. Sherman, now Professor of Dairy Industry in the New York State College of Agriculture; Udder bacteria by Alice C. Evans, now Bacteriologist in the National Institute of Health of the U. S. Public Health Service; Dissemination of disease bacteria by milk and milk products by Anne G. Benton.

Chapter XI. Metabolism and Growth of Bacteria in Milk and Milk Products. Chemistry of bacterial metabolism by W. C. Frazier, now Professor of Agricultural Bacteriology in the University of Wisconsin, revised by W. C. Frazier and G. P. Sanders; Oxygen requirements by W. M. Clark; Reducing abilities of bacteria by Anne G. Benton, revised by H. R. Curran; Phases of growth by W. R. Albus, revised by W. C. Frazier; Spores by Anne G. Benton, revised by H. R. Curran; Products of bacterial metabolism by W. C. Frazier and G. P. Sanders.

Chapter XII. Influence of Physical and Chemical Factors on Bacterial Growth. Temperature by S. H. Ayers, revised by W. C. Frazier and H. R. Curran; Electricity and ultraviolet light by Anne G. Benton, revised by J. F. Cone; Surface tension by Anne G. Benton, revised by H. R. Curran; Other factors by Anne G. Benton, revised by G. P. Sanders.

Chapter XIII. Yeasts and Molds of Milk and Milk Products. Charles Thom, now Mycologist in the Bureau of Plant Industry, U. S. Dept. of Agriculture.

Chapter XIV. Nutritional Value of Milk and Milk Products. E. B. Meigs; Colostrum by T. Swann Harding, now Editor of Scientific Publications, U. S. Dept. of Agriculture, revised by E. B. Meigs; Milk as a food for mammals past the suckling stage by W. A. Turner; Relation between the feeding of milk and anemia, iron and copper content of milk, by A. M. Hartman; Vitamins by C. A. Cary, revised by C. A. Cary and E. B. Meigs; Effect on manufacturing processes by F. M. Grant, now of the Division of Market Milk Investigations, Bureau of Dairy Industry, revised by E. B. Meigs.

Chapter XV. Physiology of Milk Secretion. E. B. Meigs and C. A. Cary; Effect of pathological conditions on composition of milk by T. Swann Harding. Chapter revised by E. B. Meigs.

Valuable advice and assistance have been given by A. C. Dahlberg, Chief in Dairy Research in the New York Agricultural Experiment Station at Geneva; by R. A. Gortner, Professor of Agricultural Biochemistry in the University of Minnesota; and by P. F. Sharp, Professor of Dairy Chemistry in the New York State College of Agriculture.

C. W. Larson, Chief of the Bureau of Dairy Industry, U. S. Dept. of

Agriculture, at the time of the publication of the first edition, gave encouragement and advice to the editorial committee and generously afforded them the facilities of the Bureau. O. E. Reed, the present Chief of the Bureau, has similarly aided the committee. The preparation of manuscript and of figures is work for which especial thanks are due to members of the Bureau staff.

In compositing the different contributions, the editors have assumed the responsibility of modifying the form of the original manuscripts in some cases in order to unify the general scheme of the book.

In citation of references, the form and abbreviations of Chemical Abstracts have been followed.

In general, Bergey's Manual of Determinative Bacteriology has been followed in the matter of bacteriological nomenclature; but, when an investigation is mentioned or discussed in the text, the names or abbreviations used by the author are copied exactly.

The Editorial Committee.

George E. Holm
E. B. Meigs
E. O. Whittier
W. C. Frazier

October 5, 1934.

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PART I

THE CONSTITUENTS OF MILK

FUNDAMENTALS OF DAIRY SCIENCE

Chapter I

Composition of Milk and Milk Products

Historical

The use of milk of animals as food by man goes far back of all recorded history. It is reasonable to suppose that the use of soured milks and of cheese as food followed very closely the use of fresh milk. The mention of milk, butter, and cheese in the earlier books of the Old Testament is in every case incidental and implies their previous use for an extended time. The use of sheep's milk probably antedated that of cow's milk.

The use of butter for food and for sacrificial purposes is mentioned in the Hindoo Vedas, which were written between 1400 B.C. and 2000 B.C. It should be mentioned to the credit of the Hindoos of that period that they valued their cows according to their yield of butter. The Scythians and Greeks and Romans did not use butter for food, but instead used it to anoint skin injuries and to apply to the hair. The soot of burned butter was supposed by them to be unusually good for sore eyes. Within the last century, butter was used in large quantities in Scotland and North England as oil for lamps.

Curd, butterfat, and whey are mentioned as the only constituents of milk by Bartolettus of Mantua in 1619,[10] but in 1628 he mentions a crude milk sugar which he obtained by evaporation of whey.[11] During the succeeding century, lactose was a very popular panacea. In India lactose was made as early as 1712.[67] Scheele[118] about 1780 proved that lactose is a true sugar. He listed the constituents of milk as butterfat, casein, lactose, a small amount of extractive substance, a little salt, and water.

That the protein of milk is partly in suspension and partly in solution, and that lactalbumin is a constituent of milk was first definitely established by Bouchardat[17] in 1857, though earlier investigations had separated the protein of milk into several fractions. Lactoglobulin was found in whey by Sebelien[121] in 1885 and its presence in whole milk and colostrum established soon after.

The presence of enzymes in milk was first demonstrated by Arnold[2] in 1881. Though it was earlier suspected that the souring of milk and

various abnormal changes in milk were of bacterial causation, it was not till 1841 that the presence of bacteria in milk was definitely proven by Fuchs.[52]

Very little is known about the history of ice cream. Water ices were made in Europe in the sixteenth century, the Crusaders probably having gotten the idea from the Saracens. Ice cream was undoubtedly a development of the water ice. Jacob Fussell, a milk dealer of Baltimore, really started the ice cream industry in the United States in 1851, though there had been a small amount of purely neighborhood retailing for some time previous. The industry has remained essentially American; there is even considerable prejudice against ice cream in some countries of Europe.

The manufacture of concentrated milks is a distinctly modern development, though the Tartars are credited with having prepared concentrated milk pastes as early as 1300 A.D. The original Borden patent for evaporated milk was applied for in 1853 and granted in 1856. Evaporated milk did not become popular until a few years later when its great utility was demonstrated by its use in the armies in the Civil War. The development of milk powder began at the same period, Grimwade's British patent of 1855 covering the first commercially usable process.

Composition of Milk

Differences in composition of milks of different species. The term milk, as commonly used, refers to the normal secretion of the mammary glands of a mammal and, unless otherwise specified, the milk is understood to be that of the cow. In various countries the milk of other mammals is used as food by man, and it is universally customary to feed infants with their mothers' milk whenever possible. The percentages of the chief constituents of milk vary to a considerable degree among the species of mammals whose milk is used as human food. In Table I only averages are given and these should be used only for comparison with averages. Milk of any species of mammal varies in composition in much the same way as does cow's milk. These variations may be studied in the publications cited in the table.

The most important characteristics of woman's milk are its low protein, low ash and high lactose percentages. The first set of values for woman's milk, in Table I, consists of averages of 1154 analyses assembled by Gardner and Fox[58] and is representative of the entire lactation period. The second set, reported by Brown, Macy et al,[10] shows only two-thirds as great a percentage of protein as the first set of figures. The reason for this may be that, for the second set of figures, the milk was not sampled until the women had been in lactation for at least two months.

An examination of Table II indicates that the ratio of non-casein protein to casein in woman's milk is nearly four times the corresponding ratio in cow's milk, and that the ratio of non-protein nitrogen to casein nitrogen in woman's milk is almost thirteen times that in cow's milk.

Table I.—Average composition of milks of certain mammals.

Species	Water	Protein *	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
Woman [53]	87.43	1.63	3.75	6.98	0.21
Woman ** [19]	87.68	1.05	4.37	6.79	0.18
Cow [92]	86.21	3.77	4.45	4.86	0.72
Cow [147]	87.90	3.13	3.65	4.50	0.72
Goat [51]	87.14	3.71	4.09	4.20	0.78
Ewe [146]	82.90	5.44	6.24	4.29	0.85
Egyptian Buffalo [95]	82.09	4.16	7.96	4.86	0.78
Chinese Buffalo [20]	76.80	6.04	12.60	3.70	0.86
Philippine Carabao [33]	78.46	5.88	10.35	4.32	0.84
Camel [8]	87.61	2.98	5.38	3.26	0.70
Mare [78]	89.04	2.69	1.59	6.14	0.51
Mare [62]	90.23	2.30	0.78	6.42	0.44
Ass [48]	89.7	2.1	1.5	6.4	0.30
Reindeer [9]	63.30	10.30	22.46	2.50	1.44

* 6.38 × nitrogen.
** Calculated from figures in article cited.

Table II.—Distribution of nitrogen in woman's and cow's milk.

Kind of milk	Total N	Casein N, as percentage of total N	Non-casein protein N, as percentage of total N	Non-protein N, as percentage of total N
	per cent	per cent	per cent	per cent
Woman's [23]	0.1749*	34.3	30.0	35.2
Cow's [28]	0.497	76.1	18.0	5.9

* Calculated from figures in article cited.

The first set of figures for cow's milk, in Table I, is from analyses made at the Illinois Experiment Station [92] and represents 1998 samples obtained during 198 whole lactation periods from 14 Ayrshire, 16 Guernsey, 19 Holstein and 15 Jersey pure-bred cows and 66 Guernsey-Holstein cross-bred cows. The averages for protein, fat and lactose are definitely higher than those in the second set of figures, which represent 208 herd samples obtained from dairy farms in New York State.[147]

Goat's milk appears to be very similar in composition to cow's milk. The averages given represent the analyses by 18 investigators of the milk of at least 326 goats.[51]

The averages for ewe's milk are from analyses of the milk of two ewes at regular intervals during the whole lactation period.[146]

Many species of mammals bearing the name of buffalo, or closely related thereto, are used as dairy animals. The milk of the Egyptian type of buffalo is used for food in many sections of southern Europe as well as in parts of Africa and Asia. The averages reported by Pappel and Hogan [95] are based on the analyses of 61 composite samples, each representing the milk of six animals. The values for the milk of the Chinese water buffalo [20] are averages of frequent analyses of the milk of 30 animals over a period of 18 months. The averages for the milk of

the Philippine carabao[33] are based on individual samples from 19 animals. Analyses of the milks of these three different species of buffaloes are included in the table because of the large variation in total solids,—principally fat. All of these milks contain a much greater percentage of fat and protein than does cow's milk.

Camel's milk is somewhat higher in percentage of fat and lower in percentage of lactose and protein than cow's milk. The averages given in Table I were computed by Barthe[8] from the reports of several analysts.

Mare's milk is used for food most commonly in parts of western Asia. It is especially low in solids, protein, fat and ash, and high in lactose. Averages found by Linton[78] for 104 samples of non-colostral milk from mares in Great Britain are higher in percentages of total solids, protein, fat and ash, and lower in percentage of lactose, than averages calculated from analyses reported by Hildebrandt.[62]

Ass's milk, much used for food in parts of Europe, is quite similar to mare's milk. The composition given is the average of the results of several analysts.[48]

Of all the natural milks used for food by man, that of the reindeer is the most concentrated.[9] The percentage of lactose constitutes an exception to this general statement, since reindeer's milk contains only about one-half the percentage of lactose present in cow's milk.

Differences in composition of cow's milk. Practically all of the milk sold in the raw or manufactured form in this country is from the cow. Even cow's milk is not a uniform article of commerce. The differences in composition, which are principally due to differing environment and inheritance,—e.g. fat,—are of such a magnitude that many dairies standardize their milk by mixing batches from different farms or breeds of cattle. It is not advisable to compare analytical results from various countries, since different methods of analysis might either obscure or exaggerate true environmental differences in the milk.

Differences in composition due to breed. Variations due to breed are of the greatest magnitude and will be considered first. The breeds represented in the United States by the greatest number of dairy cows are the Guernsey, Jersey, Ayrshire, Holstein and Shorthorn. The Shorthorn, though often considered a dual purpose breed, is included because, according to Eckles,[36] great quantities of milk are produced from grade Shorthorn cows, especially in the butter-producing states of the Mississippi valley.

The figures in Table III for the first four breeds are averages obtained by Overman, Sanmann and Wright.[93] The figures for the Shorthorn breed are averages of analyses by several investigators, reported by Eckles and Shaw.[36]

Table III shows that there is considerable difference in total solids of the milk of the Guernsey and Jersey breeds on the one hand and of that of the Ayrshire, Holstein and Shorthorn breeds on the other. More than half of this difference is in the fat percentage, the rest being mostly

Table III.—Average composition of milk of five breeds of cows.

Breed	Water	Total solids	Fat	Protein	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent	per cent
Guernsey[92]	85.13	14.87	5.19	4.02	4.91	0.74
Jersey[92]	85.31	14.69	5.18	3.86	4.94	0.70
Ayrshire[92]	86.89	13.11	4.14	3.58	4.69	0.68
Holstein[92]	87.50	12.50	3.55	3.42	4.86	0.68
Shorthorn[36]	87.43	12.57	3.63	3.32	4.89	0.73

in the protein. Except for the Ayrshire breed the lactose percentages in the milks are remarkably constant.

Differences in composition due to individuality. Within a herd of cows of a single breed there may be considerable variations in composition of milk between individuals, even though all have the same care, feed and environment. These individual differences are probably due to many factors, the principal one of which is undoubtedly inheritance. The variations are chiefly in percentages of fat and of protein; as the percentage of fat increases, the percentages of protein, ash, total solids and solids-not-fat increase, but the percentage of lactose—except in Ayrshire milk—decreases.[92]

Differences in composition between one milking and another. If the intervals between milkings are the same, the percentages of protein and lactose in milk from the same cow vary very little, but the morning milk is usually richer in fat than the evening milk, sometimes by almost 2 per cent.[37] The more frequent the milkings, the greater the variation in fat.

Variations in composition during milking. During milking, the percentage of fat increases markedly and, unless the udder is carefully stripped, the milking will not be truly representative with respect to fat. The difference between the fore milk and the strippings is only in the fat.[40] Table IV contains data which show typical variations in fat.[148]

Table IV.—Variations in fat content of milk during milking.

	Cow No. 1	Cow No. 2	Cow No. 3
	Fat per cent	*Fat* per cent	*Fat* per cent
First portion	0.90	1.60	1.60
Second portion	2.60	3.20	3.25
Third portion	5.35	4.10	5.00
Fourth portion, strippings.......	9.80	8.10	8.30

According to a preliminary report by Woodward,[153] when composite samples of whole milkings are being collected over a period of several days, it is not necessary to obtain the strippings at each milking; for the high-fat milk left in the udder at any one milking may be obtained by stripping at any subsequent milking.

Variations in composition among milks from different quarters of the udder. The work of Proks [101] shows that there are distinct differences among samples of milk from different quarters of the udder, not only in gross composition, but also in the percentage of calcium in the ash and in the composition of the fat. However, he was unable to find any regularity in these variations.

Influence on composition of successive phases of the lactation period. The secretion of the mammary glands for the first few days of lactation is known as colostrum and is quite different from the later normal secretion. Colostrum has a strong odor and a bitter taste and contains a remarkably high percentage of globulin. Table V shows the gradual change from colostrum to normal milk.[45]

Table V.—Transition from colostrum to normal milk.

Time after calving	Casein	Albumin *	Fat	Lactose	Ash	Total solids
	per cent	per cent	per cent	per cent	per cent	per cent
Immediately	2.65	16.56	3.54	3.00	1.18	26.93
10 hours	4.28	9.32	4.66	1.42	1.55	21.23
24 hours	4.50	6.25	4.75	2.85	1.02	19.37
48 hours	3.25	2.31	4.21	3.46	0.96	14.19
72 hours	3.33	1.03	4.08	4.10	0.82	13.56

* This column gives the "albumin fraction" which includes globulin. Except for a fraction of a per cent of albumin this is undoubtedly all globulin. See Chapter XIV.

The milk of the month following the colostral stage is characterized by a slightly higher percentage of albumin fraction and of fat than occurs during the long mid-stage of relatively constant composition and yield. During the final stage, when the daily quantity of milk is decreasing, the casein and fat percentages both increase somewhat.[35] For further discussion of colostrum see Chapter XIV.

Composition of milk as affected by gestation. Gestation exerts no direct influence on the composition of cow's milk, but may hasten the end of lactation, which, in itself, causes marked changes in the composition of milk.[98]

Composition of milk as affected by disease. Milk composition may undergo considerable change from the normal when the cow becomes diseased. The data on milk from diseased cows are rather discordant; they will be discussed in detail in Chapter XV.

Other factors affecting the composition of milk. The influence of kind and quantity of food on the composition of milk is discussed at length in Chapter XV. Some of the other factors which are believed to exert an influence on milk composition are weather conditions, amount and intensity of exercise, and excitement, whether due to external stimulus or to "heat." Since these influences are usually either accidental or uncontrollable they will not be discussed here.

Minor constituents of milk. The analyses of milk previously given include what may be called the major constituents of milk. Separate chapters will be devoted to the proteins, the fat, lactose, and pigments

present in milk. The products of the chemical changes which take place in milk will be treated in the discussion of the causes of these changes. There are present in fresh milk considerable numbers of organic and inorganic substances in small percentages. Some of these are of no real importance; others are of interest on account of effects which they exert out of all proportion to the quantities in which they occur.

Salts. The ash of milk is commonly understood to denote the white residue left after the milk has been incinerated at a low red heat. Since the metallic elements are in excess of the non-metallic, the ash is always alkaline in character. The composition of the ash does not represent the state of the salts as they actually occur in milk, since there is considerable alteration due to chemical reactions taking place during the incineration,—for example, oxidation. The ash contains substances derived from both the organic and inorganic compounds of the milk. The CO_2 of the carbonates is probably formed mostly from the organic constituents; the SO_3 of sulfates is regarded as a decomposition product of the milk proteins; and part of the P_2O_5 must be from the casein, since this protein contains about 1.62 per cent P_2O_5. Various investigators [50, 66, 76, 100, 141] state that from 20 to 77 per cent of the phosphates of the ash may be of organic origin. Chlorides are frequently volatilized by use of too high temperatures of ashing and consequently lost. According to Porcher and Chevallier,[100] this loss may be as much as 45 per cent. If the ash of an average milk is 0.70 per cent of the milk, the corresponding percentage of salts is approximately 0.90.

Ash analyses are given in Table VI. The values for Fe_2O_3 are much larger than those determined more recently on the milk itself without ashing, as will be seen from the following detailed discussion.

Table VI.—The composition of the ash of milk.

	Ranges of values found by several European investigators [90, 91, 107, 119, 120]	Values reported by Babcock [5]
	per cent	per cent
K_2O	17.6 to 28.8	25.02
CaO	19.9 to 28.7	20.01
Na_2O	2.6 to 11.1	10.01
MgO	1.2 to 5.0	2.42
Fe_2O_3	0.05 to 0.40	0.13
P_2O_5	21.6 to 29.3	24.29
Cl	12.2 to 16.4	14.28
SO_3	2.55 to 4.1	3.84

The trends in the variation of ash constituents during the lactation period are as follows.[138] Potassium is present in smaller quantities in colostrum than in later milk. It reaches a maximum in the second month of lactation and then declines, slowly at first and more rapidly during the last two months. Sodium decreases slightly during the first half of the lactation period and rises markedly during the second half. However, it is subject to considerable variation. Calcium is higher in colostrum

than in normal milk and remains about the same after the normal quantity has been reached until the third month before the close of lactation, when it rises slowly. Chlorine is fairly constant during the lactation period, experiencing a small constant rise throughout the period. Phosphoric acid is higher in colostrum than in normal milk, declining slowly at first and then more rapidly.

Although the ash constituents are less than 1 per cent of the milk, very slight variations in their percentages are often a source of trouble in the sterilization of evaporated milk and very probably have definite effects in the manufacture of dry milk. Citric acid, though destroyed in the ashing of milk, is an important component in milk on account of the part it plays in the salt equilibria. In the literature are found values ranging from 0.07 to 0.40 per cent, the average being probably about 0.18 per cent. Sherwood and Hammer [123] determined citric acid in 335 samples of milk from 20 cows of different breeds, in all stages of lactation, and on summer and winter rations and concluded that rations and stage of lactation do not affect citric acid percentage. Hartman and Hillig [60] analyzed 58 samples of bottled milk representing composites from large herds. Table VII summarizes representative data on salt constituents of milk.

Table VII.—The range of the salt constituents of milk.

Investigators		CaO	K_2O	Na_2O	P_2O_5	MgO	Cl	Citric acid
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Sommer and Hart [126] 30 cows	max.	0.210	....	....	0.294	0.029	....	0.312
	min.	0.132	....	....	0.193	0.019	....	0.100
Crichton [25]	max.	0.212	0.217	0.155	0.291	....	0.163	....
220 samples 20 Ayrshire cows	min.	0.126	0.148	0.050	0.166	....	0.068	....
Cranfield et al.[24]....	max.	0.225	....	....	0.305	....	....	....
672 samples 15 herds	min.	0.135	....	....	0.175	....	....	....
Sherman[122]	av.	0.168	0.172	0.069	0.240	....	0.106	....
König [71]	av.	0.161	0.177	0.059	0.189	....	0.100	....
Sherwood and Hammer [123] 335 samples	max.	....	....	....	....	....	....	0.330
	min.	....	....	....	....	....	....	0.070
Kieferle et al.[70].....	max.	....	....	....	....	....	....	0.400
104 samples	min.	....	....	....	....	....	....	0.200
Hartman and Hillig [60] 58 samples of bottled milk	av.	....	....	....	....	....	....	0.16

Salt combinations in milk. With regard to the chemical combinations and physical states in which the salt constituents of milk exist, our knowledge is not complete. The question is complicated by the fact that combinations with casein are involved, by the colloidal nature of several of the substances present, and by our lack of knowledge of the concen-

trations of most of the ions present. The lactose, citric acid, potassium, sodium, and chlorine of milk are known to be entirely in solution. The albumin, phosphates, calcium, and magnesium are partly in solution and partly in suspension. The casein and fat are entirely in suspension. Whether casein exists in combination with calcium alone, or with calcium phosphate; or whether the aggregate is a combination of calcium caseinate with calcium phosphate,—or whether sodium may be involved,—is a question that is still in doubt. It is known that both the calcium and phosphorus contents of milk decrease during the heating of milk, which would seem to prove that a part of the calcium occurs in milk as phosphate. Kay [68] lists eight forms in which he believes phosphorus occurs in milk. After all, the formulations postulated by Van Slyke and Bosworth [141] and by Söldner [125] are of questionable value, since they imply a simple status where there exists in reality a complex equilibrium that has so far not been completely or satisfactorily formulated, either from a gross heterogeneous standpoint or from the ionic standpoint. Milk equilibria are discussed in Chapter VI.

Minor inorganic constituents. In addition to the elements found in the ash in proportions greater than 1 per cent, there are present in milk small amounts of iron, copper, zinc, aluminum, manganese and iodine. The quantities of some of these can be determined by microanalytical methods. Most of these elements have been shown to be important from a physiological and nutritional standpoint. For instance, iron is necessary in hemoglobin, but copper must be present as a catalyst for its formation. Manganese is essential for stimulating growth and reproduction. Iodine is of the most importance in enabling the thyroid gland to synthesize thyroxin, and has several other useful functions.

Several investigators have made spectrographic examinations of milk ash and have found, in addition to those already listed, still other elements present in small but definite traces. Wright and Papish [154] found silicon, boron, titanium, vanadium, rubidium, lithium and strontium. Zbinden [156] found chromium, lead, tin, titanium, vanadium and occasionally germanium. Blumberg and Rask [16] found boron, barium, lithium, rubidium, strontium and titanium. The concentration of an element in the soil is probably reflected in the analysis of the milk of a cow fed on the product of that soil. The element cobalt, which has become of some nutritional interest, is apparently not present in detectable quantities in milk. Pfyl [96] has reported finding about two parts per million of silica in raw milk.

Copper. Supplee and Bellis,[132] analyzing freshly drawn milk of a large number of cows on differing rations, found the proportion of copper varying from 0.2 to 0.8 parts per million, with no apparent correlation between copper content and type of ration. Quam and Hellwig [102] found 0.26 to 0.49 parts of copper per million in cow's milk, practically the same proportion in sheep's milk, but slightly less in goat's milk. Davies [27] found from 0.05 to 0.65 parts per million in 15 samples of fresh milk. Zondek and Bandmann [158] found 0.15 to 0.20 parts per million in cow's milk and 0.5 to 0.6 parts per million in woman's milk. Elvehjem et

al.[48] analyzed 13 samples of herd milk from widely different sections of the United States and found from 0.123 to 0.184 parts per million with an average of about 0.15. They believe that some of the other figures reported are too high because of contamination of the sample with copper during analysis.

Hess and Unger[61] have shown that, if milk is contaminated with copper, the activity of the antiscorbutic vitamin is reduced. Davies[27] found that, when the copper exceeds 1.5 parts per million, milk of otherwise good quality developed an oiliness, even when properly refrigerated.

The effects of manufacturing processes on the proportions of copper and iron in dairy products have been studied by Davies[27] and by Williams.[151] They found that pasteurization and sterilization increase the percentage of both these metals in milk. The most striking effect was noted in cheese made in copper vats, the cheese containing in some cases as much as 18 parts copper per million.

Iron. Analyses of samples of cow's milk from several different sources have given results ranging from 1.0 to 4.5 parts iron per million and averaging about 2.5 parts per million.[22, 27, 42, 104] Higher values calculated from ash analyses are probably due to faulty methods of analysis. The proportion of iron in woman's and goat's milk is of the same magnitude as that of cow's milk.

Zinc. The proportion of zinc in 12 samples of market milk was found by Birckner[14] to be from 3.6 to 5.6 parts per million. Sato and Murata[113] found from 2.94 to 4.12 parts per million in normal herd milk. They found little difference in the proportions of zinc in woman's, cow's and sheep's milk. Colostral milk was found to contain about 13.00 parts per million, a marked decrease taking place during the first few days of the lactation period. Throughout the main part of the lactation period, the proportion of zinc remained constant, but increased at the end.

Manganese. The proportion of manganese in normal milk has been found to be from 0.02 to 0.06 parts per million.[69, 78, 124, 126] Sato and Murata[114] reported 0.10 to 0.21 parts per million in cow's colostrum, and 0.041 to 0.115 parts per million in sheep's milk. Kemmerer and Todd[69] found 0.082 parts per million in goat's milk.

Iodine. Iodine is present normally in milk, occurring in small but variable quantities. It is easily transmitted to milk from the feed. The amounts reported vary from 0.001 to 0.275 parts per million.[47, 79, 80, 116] Scharrer and Schwaibold[117] dried a sample of normal cow's milk and determined the distribution of the iodine. They found 4.5 per cent of the total in the fat, 31.0 per cent in the protein, 60.5 per cent in organic combination in the serum, and 4.0 per cent in inorganic combination in the serum. Milks having 1.0 to 14.0 parts iodine per million have been produced by adding organically combined iodine to the feed of the cow.[18] Organic iodine in proportions as high as 20 parts per million imparts no unpleasant taste or odor to milk. Colostral milk contains a comparatively large proportion of iodine, but this decreases to the normal amount within 36 hours after parturition.[84] Remington and Supplee[105] analyzed samples

of dried milk from several sections of the United States. 117 samples from South Carolina contained from 0.143 to 1.872 parts iodine per million, with a mean of 0.572; 9 samples from New York contained from 0.131 to 0.392 parts per million, with a mean of 0.265; 6 samples from Wisconsin contained 0.171 to 0.395 parts per million, with a mean of 0.322.

Minor organic constituents of milk. The sum of the albumin and globulin of milk varies between 0.4 and 0.8 per cent.[46, 107, 143] The average value for lactalbumin is about 0.5 per cent, this being about one-sixth of the total milk proteins. Lactoglobulin, as has been pointed out previously, may occur in colostral milk to the extent of as much as 15 per cent, but its normal percentage is about 0.05.

Some of the non-nitrogenous constituents of milk are listed in Table VIII.[80]

Table VIII.—Non-protein nitrogenous constituents of milk.

	Parts per million
Urea	10
Amino nitrogen	4.03 to 7.02
Creatin	2.2 to 2.4
Creatinin	1.1
Uric acid	1.5

Thiocyanic acid has been identified as a normal constituent of milk by Bleyer and Kallmann.[15] Choline and methyl guanidine have been reported by Mueller.[88]

Phospholipids. Lecithin, cephalin and sphingomyelin are present in milk in small quantities. They are fat-like substances, contain phosphorus and nitrogen, and are probably in colloidal condition. One of the products of lecithin decomposition is trimethylamine, a compound having a fishy odor and flavor. Table IX [85] gives a summary of the values for the percentages of phospholipids in milk and its products. The wide variations in analytical results are probably due to differences in methods.

Table IX.—The phospholipids of milk and its products.

Substance	Range of phospholipids
	per cent
Whole milk	0.0038 to 0.2889
Skim milk	0.0015 to 0.15
Cream	0.05 to 0.334
Butter	0.014 to 1.6
Milk fat	0.0 to 1.73
Buttermilk	0.0332 to 0.8768

Cholesterol. This sterol has the formula, $C_{27}H_{45}OH$. It is found associated with the fat of milk, but its function is not clearly understood. Denis and Minot [29] have determined the cholesterol in cow's milk, finding from 105 to 176 parts per million in 15 samples analyzed. They found

the quantity of cholesterol to vary in direct proportion with the total quantity of fat present. Both phospholipids and cholesterol are discussed in detail in Chapter III.

Pigments. Two pigments, carotene and lactochrome, are present in milk in very small quantities. A detailed discussion of these compounds is given in Chapter IV.

Flavoring substances. Biacetyl and related compounds are the principal substances responsible for the characteristic flavor of butter. They are probably not present in milk, but are formed during ripening and storage of cream and butter. These compounds are discussed further in Chapter III. Knowledge of the flavors of milk is in an incomplete state. The flavors of dairy products are discussed to some extent in Chapter XI.

Gases. Milk contains certain dissolved gases, chiefly oxygen, nitrogen, and carbon dioxide. Carbon dioxide is present in milk as drawn from the cow; oxygen and nitrogen are dissolved in the milk during milking or in subsequent treatments involving aeration; certain other gases are formed in milk by bacteria. Marshall [82] has investigated the gases of milk extensively and the conclusions given below and the data of Table X are from his results.

Table X.—Effect of aeration on ratio of gases in milk.

Condition of milk	CO_2	O_2	N_2
	per cent	per cent	per cent
Unexposed	81.50	2.42	16.54
After milking	59.64	13.18	27.17
After aeration over glass	40.57	20.59	38.84
After aeration over tin	35.82	20.55	44.62
After aeration over copper	42.33	17.26	40.42
After aeration through glass wool and copper sieves	25.81	23.31	50.88

(1) Milk drawn from the udder of a cow contains a high percentage of carbon dioxide and a low percentage of oxygen.

(2) During milking the percentage of carbon dioxide in the gases of milk decreases.

(3) During aeration the carbon-dioxide percentage drops still more and the oxygen percentage rises.

(4) Below a certain percentage, the elimination of carbon dioxide becomes very difficult.

(5) Most micro-organisms in milk generate carbon dioxide and absorb oxygen.

(6) Milk exposed freely to the air decreases in acidity for a time as shown by titration.

(7) Confined milk does not exhibit this decrease.

(8) This difference is largely due to evolution of CO_2 in one case and its retention in the other.

The average amount of carbon dioxide present in normal milk in the udder was found by Van Slyke and coworkers [142] to be about 10 per cent

by volume. Immediately after milking this amount dropped to 4 or 5 per cent. It dropped lower still on standing for a few hours. Milk that had been heated to the temperature of pasteurization usually had a carbon dioxide content of 3 per cent or less. They conclude that the carbon dioxide exists in milk as H_2CO_3 and probably as $NaHCO_3$.

Composition of milk serum. The serum of milk has been studied by many investigators, usually with the purpose of detecting watering by measurement of some physical attribute dependent upon the soluble constituents; such as specific gravity, refractive index, specific rotation, osmotic pressure, etc. "Milk serum" is used in the literature to indicate the clear liquid obtained from milk in any one of a variety of ways,—by rennet treatment, so-called "spontaneous" coagulation due to lactic fermentation, coagulation by acetic acid, or even by $CaCl_2$ or ethyl alcohol. While such sera have been found suitable for various physical measurements, they are known to differ considerably from the liquid menstruum of fresh milk. For purposes of analysis, to determine the nature and amount of salts in solution in milk, the serum has best been prepared by filtering fresh, sweet milk through a porcelain filter. Bialon [13] prepared sera by spontaneous coagulation, by acetic acid, and by rennet, from the same sample of milk, and found distinct though small differences in the specific gravities, due to different amounts of phosphate, calcium and protein left in solution.

Osmotic pressure can be measured indirectly as depression of the freezing point, without the necessity of freeing the liquid of the suspended material. Pins [98, 99] studied 140 samples from 35 cows, and found that the depression of freezing point varied from 0.529° to 0.569°. He could detect no correlation between these variations and the breed or age of the cow, or stage of lactation. These figures have been confirmed by a number of investigators, notably van der Laan.[74, 75] The latter made the observation that the depression of freezing point in the blood and in the milk of a cow was the same, even in cases where disease had so altered the blood that it froze at an abnormally low temperature. Disease in the udder apparently did not interfere with this equilibrium.

Jackson and Rothera [65] had previously studied the relationship between lactose content, depression of freezing point and conductivity. They showed that while the osmotic pressure of the milk depends on that of the blood, and consequently will be the same in all quarters at any milking, the conductivity and the lactose per cent may vary in the milk from different quarters, but will always be strictly reciprocal. This is well seen during recovery from pathological disturbance in one or more quarters. The alleged constancy of the "chlorine-lactose" product is dependent on this fact, as the chlorine content probably bears a fairly constant ratio to the total ionized salts. Recently the data obtained by freezing point determinations have been rendered more significant by the quantitative studies of Staub,[128] who shows that the depression must be due to three factors, —the chlorine, the sugar, and a group of residual substances. The depression due to this residual group, in milk from healthy udders, is always

equal to 0.14°, corresponding to the calculations for phosphates and citrates in true solution. In colostrum or in milk from diseased udders, this residual depression may be 0.16° to 0.20° or even greater. Staub did not identify the additional substances responsible for this abnormal value.

Comparison of milk serum with blood serum. Although the ingredients of milk must all be derived from the blood, analyses show that these two liquids differ greatly in the nature and amounts of the solutes that produce osmotic pressure. In Table XI the average results of Abderhalden's [1] analyses of bovine blood sera are compared with the average of results obtained by Van Slyke and Bosworth [141] on milk sera prepared by filtering fresh skim milk through a Pasteur-Chamberland filter.

Table XI.—Composition of milk serum and blood serum.

	Milk serum Van Slyke and Bosworth		Blood serum Abderhalden	
	Quoted	Calculated *	Calculated *	Quoted
	Grams per 1000 cc.	Grams per 1000 cc.	Grams per 1000 grams	Grams per 1000 grams
Na	0.57		3.201	
Na_2O		0.77		4.314
K	1.24		0.214	
K_2O		1.49		0.258
Ca	0.465		0.082	
CaO		0.651		0.115
Mg	0.08		0.026	
MgO		0.133		0.043
Cl	0.81			3.686
P, inorg.	0.62		0.032	
P_2O_5, inorg.		1.42		0.073
Citric acid	2.37			
Lactose	57.5			
Dextrose				1.035

* Values in this column are calculated from the values in the adjacent column headed "quoted."

Since one set of values was given in terms of grams of element, and the other was given in terms of grams of oxide, each set has been calculated to the basis of the other for convenience of comparison.

A very slight discrepancy, effective only in the third decimal place, arises from the fact that the results on milk serum were given as grams per 1000 cc. of milk. As a liter of milk serum weighs more than 1000 grams, the figures are slightly higher than they would have been if calculated entirely on a weight basis.

It will be noted that in both sera, the basic elements are in excess. Hammarsten [58] states that in blood serum a part of the bases is combined with organic substances, perhaps proteins. He quotes Rona and Takahashi who found that 25 to 30 per cent of the Ca in blood serum was non-diffusible. They also found that all the inorganic phosphorus was diffusible. In milk serum, citrates account for a part of the basic elements

(see page 22). Van Slyke and Bosworth point out that in milk, the sodium, potassium, and chlorine, as well as the citrate, are all in true solution in the serum; inorganic phosphorus, the calcium and the magnesium are partly in solution in the serum, and partly in chemical or physical combination with the suspended material. They argue that the acidity of fresh milk must be due largely to acid phosphates, since the titration values for sera are the same as those for the milk from which they were separated.

The most striking difference is the high content of sodium and chlorine in blood serum, while potassium and inorganic phosphorus are present in greater amounts in milk serum. Milk serum contains in solution all the lactose of the milk and is consequently rich in sugar, while the blood serum of a healthy animal contains only a fraction of a per cent of dextrose. It is also noteworthy that cow's blood serum, like most body fluids, contains much more sodium than potassium; while the ratio of potassium to sodium in milk is almost two to one,—a ratio very similar to that in the tissues.

Enzymes. The literature pertaining to the enzymes normally present in cow's milk is both meager and conflicting. Insufficiently controlled experiments, lack of coordination of methods and materials, use of unsuitable antiseptics, and inconsistent terminology, have all contributed to the unsatisfactory status of this subject.

The following enzymes are probably normally present in cow's milk:—protease, lactase, diastase, lipase, salolase, catalase, peroxidase and aldehydrase. These enzymes occur in milk only in very small quantities and, with the exception of protease and lipase, are probably of little or no practical significance.

Protease. The presence in milk of a proteolytic enzyme was first reported by Babcock and Russell [3] in 1897, and named "galactase." This protease brings about the slow decomposition of milk proteins into peptones, amino acids and ammonia, and is known to play an important part in the ripening of cheese.[4] Thatcher and Dahlberg [184] also demonstrated the presence of protease in normal milk, using more improved methods. Since numerous other investigators have demonstrated the presence of a proteolytic enzyme in normal milk, it seems to be fully established that protease is a normal constituent of milk, and is not of bacterial origin as sometimes claimed.

Skim milk as it comes from the separator contains a very small amount of protease, the cream contains more than the original milk, while the greatest proportion is concentrated in the slime from the separator bowl. Its presence has been demonstrated in cheese and butter, but only in very small amounts in the latter. Because of high salt content and conditions of storage it plays no part in the deterioration of normal butter.

Protease acts on casein in a neutral or slightly alkaline medium; its action is retarded in an acid medium and is inhibited, according to Thatcher and Dahlberg, by one per cent of chloroform or by 15 per cent of sodium chloride. The conditions necessary for its optimum activity

are not definitely known. It is inactivated by heat at 75 to 80°, and in acid solution is destroyed at 72° in 10 minutes.

Lactase. The presence of a lactose-splitting enzyme in milk has been reported by Stoklasa,[130] Vandevelde,[139] and others. Svanberg,[133] on the other hand, could find no evidence of this enzyme in normal milk.

Diastase. That normal cow's milk contains diastase[21, 54, 72, 77, 112, 155] (amylase) has been reported by a number of investigators, but Wohlgemuth and Strich[152] were not able to isolate it from the mammary glands and so concluded it was not normally present in cow's milk.

Koning[72] reported that 100 cc. of milk from healthy cows will decompose 0.0225 gram of soluble starch in half an hour, but found a greater diastase content in milk from diseased udders. Chrzaszcz and Goralowna[21] also reported a greater diastase content in milk from diseased udders and in colostrum up to the third day. Giffhorn[54] found that the diastase content was low in old milk, and this investigator proposed to use the diastase content of milk along with the catalase content as a criterion of the quality of milk and to detect milk from diseased udders. Diastase is probably the least variable in quantity in normal milk of all milk enzymes.

According to Chrzaszcz and Goralowna,[21] the diastase of milk is of animal origin. They found that milk diastase exhibits its maximum activity at pH 5.8—6.2 and a temperature of 30°. One hundred cc. of normal milk will completely dextrinize 0.05 to 0.1 grams of soluble starch in one hour at 30°. Diastase is inactivated in one hour at 60° to 65°, in colostrum at 65° to 70°. Cream contains more diastase than does skim milk. Colostrum and milk from diseased udders have greater diastatic activity than normal milk, and the quantity of diastase varies with the individual animal and her physiological condition. Diastase is precipitated with casein on coagulation, but a small quantity is found in the whey.

Lipase. There is much contradictory evidence concerning the existence or nonexistence of a true lipase in normal milk. Vandevelde,[139] Koning,[72] and Vincent[144] were unable to prove the presence of a fat-splitting enzyme in milk. Thatcher and Dahlberg[134] found evidence of a lipase in only one sample of butter stored at 40° for four days. Palmer,[94] after reviewing the literature, carried out experiments which failed to show any evidence of its presence in normal cow's milk. In conflict with these findings, several investigators have been able to demonstrate a true fat-splitting lipase identified with milk. Moro[87] employed olive oil as a substrate and found that milk was able to induce hydrolysis. Rogers[108] found lipase to be the cause of increased acidity in canned butter on standing. Later, Rogers, Berg and Davis[109] showed that cow's milk is able to hydrolyze ethyl butyrate under the conditions employed and that the activity is greatly weakened if milk is heated to 66°, and is destroyed at 80°.

What seems to be final proof of the presence of lipase in milk was supplied by Rice and Markley[106] in experiments wherein high-fat creams

as substrate were saturated with sucrose, which served as a preservative. The titration value of the medium was taken as a measure of hydrolysis. Nair [89] confirmed this work, using a similar method of analysis.

Salolase. The presence of salolase in normal cow's milk has been reported by Vandevelde.[139] In preliminary experiments, Grimmer [55] was unable to obtain it from the extracts of glands of cows, but succeeded in obtaining it from the extracts of the mammary glands of sheep, goats, swine and mares. Subsequently, he secured positive results with cow-gland extracts.[56]

Catalase. The presence of catalase in normal cow's milk was first demonstrated by Raudnitz.[103] That milk catalase is a secreted enzyme has been shown by Grimmer,[56] Harden and Lane,[59] and others.

The quantity of catalase in milk varies with the breed, with the individual animal, and with different intervals between milkings, there being most in the last portion drawn. However, milk containing a higher percentage of fat does not necessarily contain more catalase. Colostral milk and milk from cows in the last stages of lactation contain large amounts of catalase. The feeding of green stuffs increases the catalase content of milk.

Cream contains a greater proportion of catalase than the milk from which it is separated, and a still greater proportion is to be found in the separator slime. An increased catalase content accompanies appreciable numbers of bacteria or leucocytes in milk. For this reason the catalase content of milk has been proposed as a means of determining the quality of milk and of detecting milk from diseased udders. Thatcher and Dahlberg [134] were able to demonstrate the presence of catalase in butter, although it is true that the greater part of the catalase remains in the buttermilk. On coagulation of milk, the catalase is precipitated with the casein, and cheese has been shown to contain a large amount of catalase. The amount varies, however, with the type of cheese, this indicating that bacteria must be the source of a considerable portion of the cheese catalase.

Normal milk contains a definite amount of catalase and, according to Koning,[72] 100 grams of fresh normal milk will decompose as much as 110 milligrams H_2O_2 in two hours. The maximum activity of this enzyme at 0° occurs at pH 6.8 to 7.0 (Balls and Hale [6]). Its activity is retarded in acid solutions. Heating milk for half an hour at 65° to 70° completely destroys the catalase and its activity is greatly impaired if it is heated for the same length of time at lower temperatures. This fact is utilized in determining whether or not milk has been heated.

Peroxidase. Peroxidase is an inherent enzyme of milk, as shown by Grimmer,[55] Koning,[72] Harden and Lane,[59] Violle [145] and others. All milk contains peroxidase, but the amount present is somewhat variable. The greatest concentration recorded has been found in separator slime. Violle found a relatively large quantity of peroxidase in milk from diseased glands. Thatcher and Dahlberg [134] reported small amounts of peroxidase present in butter.

This enzyme liberates active oxygen from H_2O_2 and it is by this

activity that its presence is demonstrated, guaiaconic acid being oxidized to a blue substance by the active oxygen.

According to Zilva,[157] the effect of heat on peroxidase is too slight to be detected in milk heated below 70° and so a test for peroxidase could not be used to detect pasteurization. Koning [72] found that small proportions of alkali accelerate inactivation, while small proportions of acid retard inactivation. Balls and Hale [7] found horse-radish peroxidase most active at a temperature of 30° and at pH 6.8 to 7.0 with pyrogallol as substrate; milk peroxidase undoubtedly has very similar or identical properties.

Aldehydrase. The presence in cow's milk of an oxidase sometimes known as Schardinger's enzyme has long been known.[115] In the presence of a suitable oxidant this enzyme catalyzes the oxidation of aldehyde. This enzyme was found in milk by Stetter,[120] Koning,[72] Harden and Lane [59] and others, all of whom called it reductase because of its ability to reduce methylene blue to its leuco base in the presence of an aldehyde. It is believed to be more abundant in fat and in cream than in skim milk and, according to Koning, is not present in separator slime. It is relatively more abundant in the last part of the milking and in milk from diseased udders. It is not destroyed by heating for one-half hour at 65°; Piccard and Rising [97] showed that it is active up to the coagulating temperature of albumin, 72° to 80°. These authors also report that reductase remains after the fat and casein have been removed from milk, but that it is precipitated by strong acids.

Morgan, Stewart and Hopkins [86] in 1922 found in milk an enzyme capable of oxidizing the purine bases, hypoxanthine and xanthine, to uric acid. Shortly afterwards, Haas and Hill [57] reported the discovery in milk of adenase and a nitrate-reducing enzyme active in the presence of an aldehyde. Subsequently, Dixon and Thurlow [31] confirmed both these findings, but also presented evidence which strongly suggested that the xanthine oxidase, adenase and nitrate-reducing enzyme are identical with the aldehyde-oxidase. In the nitrate-reducing reaction, which occurs only in the presence of an aldehyde, these authors showed that the aldehyde acted as the reducing substance, the aldehydrase as catalyst, and the nitrate as the oxygen donor.

Opposed to the view that aldehydrase and xanthine oxidase are identical is the more recent work of Wieland and his coworkers.[148, 149, 150] They showed that the promoting activity of methylene blue for oxidation of xanthine is not in constant proportion to the promoting activity of methylene blue for oxidation of aldehyde. They were able also to separate the two enzymes by selective adsorption. These results supported by other reactions have led these workers to conclude that two separate enzymes are involved. From this it will be seen that the number of enzymes concerned in these reactions is still unsettled.

Vitamins. All of the recognized vitamins are generally considered to be present in milk. The quantities of vitamins A and B in milk are comparatively large; the other vitamins are present in small but quite definite

quantities. Exposure of milk to ultraviolet light is known to increase its antirachitic effect, and large quantities of milk are treated in this way commercially. Extensive discussion of vitamins in milk and its products will be found in Chapter XIV.

Composition of Milk Products

General discussion. The principal feature of the manufacture of nearly all commercial milk products is essentially the separation of one or more constituents from milk. Either the substances separated or the remaining mixture or both may be products of value. This separation is rarely sharp; so that practically all milk products contain all the constituents of milk, but with the proportions of one or more considerably altered. For example, in the concentration of whole milk the alteration is in the ratio of water to the other components. By means of the cream separator, on the other hand, are obtained two products of value,—the cream, containing practically all of the milk fat and varying proportions of "fat-free" milk, and the skim milk, containing the bulk of the "fat-free" milk and very small quantities of milk fat. Both cream and skim milk may be concentrated by removal of water, but, even after these two operations, the relative proportions of protein, lactose and salts are practically the same in both products as in the original milk. Cream from the separator may be churned to produce butter, which differs analytically from concentrated cream in that solids-not-fat have been removed in addition to water. The buttermilk from sweet cream butter is practically the same in composition as skim milk. Butter may be melted and the butter oil separated, which represents what may be considered the final step in the concentration of milk fat. The manufacture of cheese is essentially the preparation of a relatively impure casein containing larger or smaller quantities of fat, depending on whether the milk has been wholly or partially skimmed. In the cheese-making process, the proportions of all the milk constituents are changed relative to one another. Lactose may be in part removed from cheese whey by crystallization, leaving a mixture of lactose, albuminous protein and salts in the mother liquor. In contrast to cream, butter, casein and lactose, which are essentially concentrated single constituents, such products as ice cream and sweetened condensed milk are made by the addition of considerable quantities of substances foreign to milk. In a few products, such as cheese and cultured buttermilk, certain of the milk constituents are chemically changed by bacterial action.

The changes in composition which occur in dairy manufacturing processes have been pointed out in order that it may be clear why each milk product resembles milk in some respects, from the analytical standpoint, but not in others.

Any single product is likely to vary considerably, its composition depending on the composition of the raw milk and on the method of manufacture as determined by the aims of the producer or the desires of his

customers. Consequently it is neither possible nor desirable to give a single analysis as a standard for any product. The effort has been, in compiling the following tables, to give an idea of the usual range of composition. In a few cases, because of lack of sufficient data or because of other difficulties, this method has not been feasible.

Evaporated whole milk. This product is made by concentrating whole milk under vacuum at a temperature of 54° to 60°. The concentrated product is transferred to small cans and sterilized under pressure by live steam to kill organisms and destroy enzymes. Water and gases are the only substances removed; nothing is added. The values given in Table XII were calculated from the analyses of 20 samples reported from the Connecticut Experiment Station at New Haven in 1919.[181]

Table XII.—The composition of evaporated whole milk.

	Water	Protein	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
Maximum	74.94	7.91	9.57	11.39	1.75
Minimum	71.29	6.38	7.67	8.10	1.34
Average	73.40	6.54	8.24	9.93	1.54

Dried whole milk. Whole milk and other liquid milk products are dried usually by either the spray or the drum process. The milk may be dried in one operation or may be partially concentrated before drying. The degree of solubility of dry milks is of considerable importance and varies inversely with duration and temperature of the drying process. Methods of analysis of dry whole milk are at the present time neither satisfactory nor uniform. The values in Table XIII represent a composition which may be expected under present standards for manufacture.[40]

Table XIII.—The composition of dry whole

Water	Protein	Fat	Lactose	
per cent	per cent	per cent	per cent	per cent
4.0	27.2	26.0	36.8	6.0

Sweetened condensed milk. This product is a concentrated whole milk preserved by the addition of sucrose instead of by sterilization. Table XIV is a summary of the analyses of 31 samples reported from the Connecticut Experiment Station at New Haven in 1919.[181]

Table XIV.—The composition of sweetened condensed whole milk.

	Water	Protein	Fat	Lactose	Ash	Sucrose
	per cent	per cent	per cent	per cent	per cent	per cent
Maximum	31.72	8.68	10.17	15.76	2.08	46.02
Minimum	23.61	7.27	8.23	10.42	1.56	30.14
Average	26.75	7.85	8.99	12.94	1.77	40.59

Cream. Cream consists essentially of the fat of milk together with a portion of the other solids and of the water of milk. It has been demonstrated repeatedly that the ratio of water to solids-not-fat in cream is the same as that in the milk from which the cream has been produced. Cream may contain from 10 to 70 per cent fat, the percentage depending upon the speed of the separator, the temperature of the milk, the rate of milk in-flow and the setting of the cream-screw. Cream with a fat percentage greater than 70, "plastic cream," has been produced by running the cream through the separator repeatedly until the required fat concentration was secured. It is the usual practice in the United States to offer three grades of cream,—light, heavy and whipping,—containing approximately 20, 30 and 40 per cent of fat, respectively. Table XV gives calculated analyses of each grade of cream and the analysis of the milk on which the calculations are based.

Table XV.—The composition of cream.

	Water	Protein	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
Milk	87.30	3.55	3.62	4.82	0.71
Light cream	72.46	2.95	20.00	4.00	0.59
Heavy cream	63.41	2.58	30.00	3.50	0.52
Whipping cream	54.35	2.21	40.00	3.00	0.44

Dried cream. A small quantity of desiccated cream is manufactured in the United States, usually by the spray process. In this product the ratio between the fat and the solids-not-fat is the same as that of the cream previous to drying. The analyses in Table XVI are from the circular of a prominent manufacturer.

Table XVI.—The composition of dried cream.

Sample No.	Water	Protein	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
1	0.80	19.19	50.40	25.45	4.16
2	0.66	13.42	65.15	17.86	2.91
3	0.56	11.12	71.15	14.74	2.43

Skim milk. The percentages of all constituents of skim milk, except of course the fat, are proportionally greater than in the whole milk from which it has been made. The analyses in Table XVII are averages calculated from data of several authorities.

Table XVII.—The composition of skim milk.

	Water	Protein	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
Separator skimmed	90.35	3.72	0.15	4.98	0.80
Hand skimmed	90.25	3.58	0.75	4.66	0.76

Evaporated skim milk. This concentrated unsweetened product is usually marketed unsterilized and packed in ten-gallon cans. It is known to the trade as plain condensed bulk milk. Its composition varies widely, depending on the composition of the skim milk used and on the condensing ratio employed. Analyses are not available. The composition in Table XVIII is calculated from the figures given in Table XVII for separator skimmed milk on the assumption of a three to one condensing ratio.

Table XVIII.—The composition of evaporated skim milk.

Water	Protein	Fat	Lactose	Ash
per cent 71.05	per cent 11.16	per cent 0.45	per cent 14.94	per cent 2.40

Sweetened condensed skim milk. This is a concentrated skim milk preserved by the addition of sucrose. Table XIX is compiled from analyses of six samples reported from the Connecticut Experiment Station at New Haven in 1919.[181]

Table XIX.—The composition of sweetened condensed skim milk.

	Water	Protein	Fat	Lactose	Ash	
	per cent	per cent	per cent	per cent	per cent	per cent
Maximum	30.16	10.05	1.67	27.02	2.34	48.22
Minimum	26.32	8.29	0.61	13.06	1.71	30.42
Average	28.74	9.14	1.01	18.18	2.05	40.88

Concentrated sour skim milk. This is a product made from skim milk which has been pasteurized and then soured by means of a pure culture of lactic bacteria before concentrating, or from a mixture of skim milk and whey pasteurized and soured separately before combining and concentrating. Table XX gives the approximate composition which may be expected for these products.[110]

Table XX.—The composition of concentrated sour skim milk.

	Water	Protein	Fat	Lactose	Ash	Acid as lactic
	per cent	per cent	per cent	per cent	per cent	per cent
From skim milk.....	72.00	10.19	0.17	9.43	2.13	6.08
From skim milk plus whey	69.69	8.26	0.20	13.46	2.54	5.85

Dry skim milk. This is the product resulting from the removal of most of the water from skim milk and contains not more than 5 per cent moisture.[49] The percentages in Table XXI represent a composition which may be expected under present standards of manufacture.

Table XXI.—The composition of dry skim milk.

Water	Protein	Fat	Lactose	Ash
per cent	per cent	per cent	per cent	per cent
4.0	37.4	1.0	49.2	8.4

Butter. The differences in the composition of butter are due almost entirely to differences in methods of manufacture. The limits of variation are gradually becoming narrower due to the enforcement of Federal and State standards and to more nearly uniform methods taught by the State dairy schools. The tendency is for the average percentages of milk fat and water to approach more closely the standards of a minimum of 80 per cent for fat and a maximum of 16 per cent for water. More interest is now being shown in the minor constituents of butter, such as the metals, the phospholipids, the lactates, etc. Recent unpublished work of the Research Laboratories of the Bureau of Dairy Industry indicates that the percentage of lactic acid and lactates in butter varies considerably and ranges from 0.01 to 0.20 per cent, expressed as lactic acid. Table XXII gives values for the composition of 2919 samples of creamery butter.

Table XXII.—The composition of butter.

		American creamery butter [187]	Minnesota creamery butter [88]	Iowa creamery butter [26]
Date reported		1912	1925	1932
Number of samples		695	1363	861
		per cent	per cent	per cent
Fat	Range	76.5-87.4	78.5-86.5	77-84
	Average	82.41	81.55	80.85
Water	Range	10.0-16.8	11.25-18.25	13-19
	Average	13.90	15.20	15.88
Curd	Range	0.2-3.4	....	1-2
	Average	1.18	0.98	0.89
Salt	Range	0.7-6.0	....	1-4
	Average	2.51	2.29	2.39

Butter oil. Commercial butter oil is a purified milk fat, made usually by centrifuging melted butter, and used in the baking and ice cream industries. Butter oil made from unsalted sweet-cream butter is a very pure product containing only minute traces of water and gases held in solution. Their presence can be demonstrated only by the use of high vacuum.

Buttermilk. Genuine buttermilk,—the liquid phase found in the churn after the process of churning is complete,—varies in composition depending on the composition of the serum of the milk from which it is derived, on the acidity of the cream churned, and on the technic of churning. Table XXIII gives the composition of two samples, one from sour cream and the other from sweet cream.[107]

Table XXIII.—The composition of genuine buttermilk.

	Water	Protein	Fat	Lactose	Ash	Lactic acid
	per cent	per cent	per cent	per cent	per cent	per cent
From sour cream....	91.61	3.30	0.50	3.40	0.65	0.50
From sweet cream...	90.98	3.51	0.35	4.42	0.73	0.01

Commercial buttermilk. The product sold for beverage purposes under the name of buttermilk is usually skim milk which has been first pasteurized and then inoculated with cultures of lactic and other bacteria selected to give it the desired acidity, flavor and aroma. Granules of butter are sometimes added directly to the cultured milk, or the cultured product containing some milk fat may be subjected to the churning process in order that it may more closely simulate genuine buttermilk. The composition is the same as that of a true buttermilk derived from sour cream, the acidity usually ranging from 0.5 to 0.8 per cent lactic acid. In case a culture of *Lactobacillus bulgaricus* is used either alone or in mixed culture with *Streptococcus lactis,* the acidity of the finished product may be as high as 1.0 to 2.0 per cent lactic acid.

Acidophilus milk. This is a therapeutic product made from sterilized skim milk by inoculation with a selected culture of *Lactobacillus acidophilus.*[111] It is essentially a buttermilk in composition, but may contain considerable quantities of lactose added for therapeutic advantages.

Condensed buttermilk. This product is generally salvage from the manufacture of butter in creameries of large capacity. It is used almost entirely as stock feed. Table XXIV gives the analysis of three samples.[61]

Table XXIV.—The composition of condensed buttermilk.

Sample No.	Water	Protein	Fat	Lactose		
	per cent	per cent	per cent	per cent	per cent	per cent
1	69.0	10.8	2.3	12.0	2.2	4.5
2	73.5	9.0	1.0	9.8	1.7	5.0
3	70.8	9.9	1.3	11.7	2.0	4.3

Dried buttermilk. Some large creameries, instead of condensing their buttermilk, dry it by either the spray or drum processes. Small quantities of dried buttermilk are sold to bakers and to manufacturers of prepared foods, but most of this product is used in stock feeds. Table XXV gives the composition of a dry buttermilk calculated from the analysis of the sour cream buttermilk given in Table XXIII.

Table XXV.—The composition of dry buttermilk.

Water	Protein	Fat	Lactose	Ash	Lactic acid
per cent	per cent	per cent	per cent	per cent	per cent
1.93	38.74	5.87	39.91	7.68	5.87

Cheese. Cheese is a complex food product consisting mainly of coagulated casein, fat and water. Lactose is practically never present in a well-ripened hard cheese. The milk used in cheese manufacture may have its original fat content, or may have been skimmed to varying degrees, or even have been enriched by the addition of cream before curdling. The method of manufacture influences considerably the composition of cheese. Although a list of the varieties of cheese manufactured in the various countries of the world would contain several hundred different names, the number of distinct types is much smaller. Only 10 of the more important types of cheese manufactured or sold in the United States are considered here. They are listed in approximately the order of their hardness when ready for consumption, as follows:—Parmesan, Swiss, Cheddar, Roquefort, Brick, Limburger, Camembert, Neufchatel, Cream and Cottage.

Very hard cheeses, with gas holes. Parmesan is a large, Italian-type cheese made from partially skimmed milk. After the making and salting process, which takes about 40 days, the cheese is stored in cool, well-ventilated rooms for several years, being rubbed occasionally with oil. Parmesan becomes very hard and must be grated before using. The values in Table XXVI are calculated from the analyses of 8 ripe Parmesan cheeses.[81]

Table XXVI.—The composition of Parmesan cheese.

	Water	Protein	Fat	Ash	Lactic acid
	per cent	per cent	per cent	per cent	per cent
Maximum	36.11	49.44	22.83	7.18	2.92
Minimum	30.20	39.12	12.58	5.20	1.68
Average	32.17	44.31	18.94	6.29	2.36

Swiss cheese is made from heated and pressed curd obtained by the action of rennet on partially skimmed or whole milk, and is ripened by special gas-producing bacteria which cause characteristic eyes or holes.[49] The milk is usually standardized to a definite fat-casein ratio. This type of cheese was made originally only in Switzerland but, as a result of the wide emigration of the Swiss cheese-makers, it is now made in many parts of the civilized world. Table XXVII [186] gives the range of composition of high-grade domestic Swiss cheese. More recent analyses indicate that 33 to 36 would be a more nearly correct range for percentage of water.

Table XXVII.—The composition of domestic Swiss cheese.

Water	Protein	Fat	Ash	Salt
per cent 30-34	per cent 26-30	per cent 30-34	per cent 3-5	per cent 1-1.4

Very hard cheese, without gas holes. The cheese known as Cheddar, American or American Cheddar is made from heated and pressed

curd obtained by the action of rennet on partially skimmed or whole milk. Usually the fat-casein ratio of the milk is standardized. Cheddar is probably the most important cheese made in English-speaking countries, from the standpoint of quantity. It is marketed in many different sizes and eaten at many different stages of ripeness. Table XXVIII gives values from 150 analyses of green cheese made in this country, and from 414 green cheeses made in Canada more recently.[63, 140]

Table XXVIII.—The composition of American Cheddar cheese (green).

	Water	Protein		Lactose, ash, etc.
	per cent	per cent		per cent
United States, 1892-1893				
Maximum	43.89	26.11	36.79	7.02
Minimum	32.69	20.80	30.00	3.12
Average	36.84	23.72	33.83	5.61
Canada, 1925-1926				
Maximum	42.06	....	39.96	
Minimum	25.74	....	29.17	
Average	34.82	....	33.75	

Semi-hard cheeses, ripened by molds. Genuine Roquefort is a rennet cheese made in southern France from unskimmed sheep's milk. The unheated and unpressed curd is inoculated with a special mold,—*Penicillium roqueforti,*—and ripens with the growth of the mold. The fully ripened cheese is friable and has a mottled appearance in section. A cheese of the same type is made from cow's milk in the United States. Other well-known rennet cheeses characterized when ripe by a green mold throughout the cheese are the Gorgonzola of Italy and the Stilton of England, both made from cow's milk. The figures in Table XXIX are from the analyses of a group of selected, high-grade, genuine sheep's-milk Roquefort.[84]

Table XXIX.—The composition of Roquefort cheese.

	Water	Protein	Fat	Ash	Salt
	per cent	per cent	per cent	per cent	per cent
Maximum	40.10	23.06	33.53	6.81	4.88
Minimum	37.49	19.14	31.50	5.18	3.64
Average	38.69	21.39	32.31	6.14	4.14

Semi-hard cheese, ripened by bacteria. Brick cheese is a rennet curd, whole milk cheese made mostly in the United States, intermediate in type between Swiss and Limburger. Similar cheeses of foreign make are Port du Salut, Oka and Münster. Table XXX is calculated from the analyses of 4 samples of Brick cheese.[82]

Soft cheeses, ripened by mold. Camembert is another mold-ripened cheese which originated in France, but which is now made also in the United States. The mold remains on the rind of the cheese and secretes

Table XXX.—The composition of Brick cheese.

	Water	Protein, amids, etc.	Fat	Ash
	per cent	per cent	per cent	per cent
Maximum	45.26	23.29	33.77	4.20
Minimum	39.61	20.03	28.34	1.68
Average	42.47	21.05	30.66	2.98

enzymes which penetrate the body of the cheese to carry out the ripening process. Probably because of the shorter ripening period, the domestic Camembert is usually more moist than the typical foreign cheese. Other well-known cheeses of this type are Brie and Ripened Neufchatel. Table XXXI contains figures from the analyses of 10 samples of imported and 2 samples of domestic Camembert.[185]

Table XXXI.—The composition of Camembert cheese.

	Water	Protein	Fat
	per cent	per cent	per cent
Maximum	54.41	21.80	32.13
Minimum	43.08	16.83	23.04
Average	47.91	19.66	27.33

Soft cheese, ripened by bacteria. Limburger is a rennet curd cheese made from cow's milk, usually unskimmed, and ripened by bacteria. The ripening process takes about two months. Table XXXII gives the usual range of composition.[186]

Table XXXII.—The composition of Limburger cheese.

Water	Protein	Fat
per cent	per cent	per cent
38-44	21-25	25-30

Soft cheeses, unripened. To avoid spoilage and loss by action of molds and bacteria, this class of cheeses must be made from good-quality milk, must be kept in storage at low temperatures, and should be marketed during a comparatively short period after manufacture. Domestic Neufchatel is made from completely skimmed, partially skimmed, or whole milk. Lactic organisms are employed for flavor production, but rennet is used for coagulation. The range of composition given is for whole milk Neufchatel. Cream cheese differs from whole milk Neufchatel in that the milk used is exceptionally high in fat or its fat content is increased by the addition of cream. Cottage cheese is essentially a skim milk cheese, although in some factories cream is added after the curd is separated. The curdling is usually accomplished by lactic organisms and the curd is not worked, as in the case of Neufchatel and cream cheese.

Table XXXIII.—The composition of soft cheeses.

	Water	Protein		Ash
	per cent	per cent	per cent	per cent
Domestic Neufchatel[83] ..	50.0-55.0	18.0-21.0	23.0-28.0	0.5-1.25
Cream[83]	38.0-43.0	13.0-16.0	43.0-48.0	0.5-1.25
Cottage[41]	71.4-79.9	12.7-21.1	0.4- 1.9	0.2-1.1*

* Salt free ash.

Whey. This is the product remaining after the removal of most of the casein and fat from milk in the process of cheese making. Whey varies considerably in composition depending on the composition of the original milk, on the cheese-making process of which it is a by-product, and on the skill of the cheesemaker. Fresh whey contains most of the salts, lactose and albumin of the milk, and, in addition, other nitrogen compounds including traces of casein and a little milk fat. Storage of whey for any considerable time results in a decrease in the percentage of lactose, an increase in the acidity, and breakdown of the nitrogen compounds. A small quantity of whey is used as a commercial source of lactose. It is used as a stock feed either directly or as the condensed or dried product. Table XXXIV gives the range of composition of a large number of samples of Cheddar cheese whey.[12]

Table XXXIV.—The composition of Cheddar cheese whey.

Water	Solids	Protein	Fat	Lactose	Ash	Acid as lactic
per cent	per cent	per cent	per cent	per cent	per cent	per cent
92.87	6.57	0.82	0.12	4.62	0.366	0.144
to	to	to	to	to	to	to
93.43	7.13	0.95	0.36	5.01	0.649	0.236

Whey butter. Where the volume of whey handled and the percentage of fat present are sufficient to pay the cost, the whey is run through a separator. The cream produced is ripened with a bacterial culture and churned, producing a butter practically identical in composition with ordinary butter.

Whey cheese. From wheys in which the albumin has not been coagulated, a whey cheese may be made by either of two processes. In the first, the whey is concentrated by boiling until it has, on cooling, a firm sugary consistency. In the second, the albumin is coagulated by heat and acid and the curd skimmed off and pressed in hoops. The first process produces Mysost or Primost; the second, Ricotte or Ziger. Table XXXV gives the approximate range of composition of these cheeses.[32]

Table XXXV.—The composition of whey cheese.

Kind	Water	Protein	Fat	Lactose	Ash
	per cent	per cent	per cent	per cent	per cent
Mysost	10.0-38.0	6.3-14.0	1.2-34.5	30.8-61.4	3.3-6.4
Ziger	68.5-74.7	15.9-22.1	3.1- 5.2	3.9- 4.0	2.0-3.6

Condensed whey. This is not a common commercial product. That produced is usually used as a constituent of stock feed. A hitherto unpublished analysis is as follows: Water, 48.1 per cent; protein, 7.5 per cent; fat, 2.4 per cent; ash, 5.6 per cent; lactose, 21.4 per cent.

Dried whey. Whey may be dried by any of the processes used for drying milk. No analyses of dried whey are available. However, its composition may be calculated from that of whey by allowing a total solids percentage of 95.0 to 98.5 in the dried product.

Soluble whey protein powder. This substance is a dried whey from which part of the lactose has been removed by crystallization previous to drying. Table XXXVI contains hitherto unpublished analyses of this product.

Table XXXVI.—The composition of soluble whey protein powder.

	Water	Protein	Lactose	Ash
	per cent	per cent	per cent	per cent
From Swiss cheese whey				
Maximum	5.05	44.46	52.00	18.15
Minimum	1.62	32.45	37.20	12.45
Average	3.28	38.02	43.02	16.38
From Roquefort cheese whey				
One sample	3.3	24.7	46.5	25.5

Commercial casein. Casein is made almost entirely from skim milk, precipitation being accomplished either by the use of rennet or by acidification. Differences in composition of casein are caused by differences in the composition of the skim milk, in the type of process, and in the care with which the precipitation and washing of the curd are accomplished. It is not feasible to give averages, since values vary so much within their range and since methods of analysis are not uniform. The percentage of moisture of all types of casein usually falls within the range 3.0 to 8.0; that for fat, within the range 0.0 to 1.0. The percentages of fat in two caseins should not be compared, unless it is known that the methods of determination were the same. Rennet casein consistently contains between 7.0 and 8.3 per cent ash, this comparatively large value being accounted for by the fact that rennet casein is essentially a calcium caseinate. Discussion of the ash percentages of acid-precipitated caseins is complicated by the little appreciated facts that neglect to add an oxidizing agent to the sample before ashing causes values to be low, and that, if a casein containing less than 2.5 per cent ash is incinerated without addition of a metal salt of an organic acid, varying proportions of P_2O_5 are volatilized. Pure casein contains 1.62 per cent of P_2O_5 in organic combination, and for this reason values of ash percentages less than 2.5 per cent are open to question, unless it is known that precautions were taken, in the determination, to fix the P_2O_5. Values less than 1.62 per cent should be disregarded unless it is known both that the P_2O_5 was not volatilized and that 1.61 was subtracted from the determined percentage to give what is called in some European journals "true" ash. What may

be called "total" ash of natural-sour casein falls usually within the range 2.5 to 4.5 per cent; of hydrochloric and sulfuric casein, 3.0 to 5.5 per cent; and of grain-curd casein, 1.9 to 3.0 per cent.

Commercial lactose. Lactose is made in this country most generally from grain-curd casein whey, although a fresh cheese whey may be used. The values given in Table XXXVII are from analyses [41] of 17 lots of 300 barrels each. Fourteen lots were of domestic manufacture, the other three of foreign. In all the samples the lactose was not less than 99.73 per cent as determined by the polariscope.

Table XXXVII.—The impurities of commercial lactose.

	Nitrogen	Fat	
	per cent	per cent	per cent
Maximum	0.0180	0.0250	0.0420
Minimum	0.0037	0.0016	0.0060
Average	0.0098	0.0102	0.0209

Ice cream. Since commercial ice cream practically always contains several different milk products mixed in order to standardize the ice cream to a predetermined composition based in turn partly on local legal requirements and partly on consumer preference, it is not practicable to give a truly typical analysis. The range of composition of wholesale, commercial ice creams, exclusive of added fruits, nuts and other flavoring materials, is given in Table XXXVIII.

Table XXXVIII.—The composition of ice cream.

	Fat	Milk solids -not-fat	Cane sugar		Egg solids
	per cent	per cent	per cent	per cent	per cent
Maximum	20	14	17	0.50	1.0
Minimum	8	6	13	0.00	0.0
Usual range	10 to 14	9 to 11	14 to 16	0.25 to 0.50	0.3 to 0.6

Malted milk. This is a product made by combining whole milk with the liquid separated from a mash of ground barley malt and wheat flour, with or without the addition of sodium chloride, sodium bicarbonate and potassium bicarbonate, in such a manner as to secure the full enzymic action of the malt extract.[49] The malted mixture is then carefully re-

Table XXXIX.—The composition of malted milk.

	Water	Protein	Fat	Nitrogen-free extract	Fiber	Ash
	per cent	per cent	per cent	per cent	per cent	per cent
Maximum	5.93	15.38	8.36	74.49	0.30	4.00
Minimum	2.03	12.94	4.11	68.79	0.00	3.08
Average	4.20	14.16	6.54	71.51	0.16	3.41

duced to a dry powder. The values given in Table XXXIX are calculated from analyses [181] of 7 samples representing 4 brands of malted milk.

Fermented milks. In addition to the soured milks already discussed, there is a type of fermented milk which contains both lactic acid and alcohol. Kefir and koumis [111] are examples of these products. They may contain as much as 3.0 per cent alcohol and as much as 2.5 per cent lactic acid, but lower values are more usual. A portion of the protein is partially hydrolyzed.

Separator slime. In the separation of cream, a light gray substance collects on the inside of the bowl of the separator. This usually contains 66 to 72 per cent water, 18 to 26 per cent protein, 0.5 to 3.0 per cent fat, 2.5 to 3.5 per cent ash, and 2.0 to 8.0 per cent other organic matter. Bacteria and leucocytes are highly concentrated in this slime and apparently several substances enzymatic in nature are also present in concentrations much higher than in ordinary milk.

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Chapter II

Proteins of Milk

Introduction

Proteins are compounds of very great importance in biochemistry since they enter into the human and animal food supply to such a large extent and form such a large percentage of body substance. The chemical investigation of these substances is extremely difficult on account of their complex structure, the fact that most of them do not crystallize, and the ease with which they undergo decomposition or other chemical change, when subjected to the usual methods of investigation.

Casein (or caseinogen, as it is termed by the British) is the principal protein of milk, occurring therein as a calcium compound and forming, in the case of cow's milk, nearly 3 per cent of the whole. It belongs to a very complex group of proteins,—the phospho-proteins. On account of its ready availability and its importance, it has been investigated to a greater extent than any other protein. Lactalbumin occurs in milk, forming about 0.50 per cent of cow's milk. Lactoglobulin, another milk protein, forms about 0.05 per cent of cow's milk. Other proteins are probably present in milk, but in very much smaller proportions than these mentioned.

Chemistry of Casein

Elementary composition. Typical elementary analyses of the casein of cow's milk are given in Table XL. Elementary analyses of the caseins of milks of other mammals [15, 109, 180] show no significant differences from those of cow's milk casein. The comparative constancy of ultimate composition has been used in the past as the basis for the calculation of an empirical formula for casein on the assumption that casein is a definite single chemical species. This assumption has lately been

Table XL.—Elementary analyses of casein from cow's milk.

	Hammarsten [47]	Lehman [53]	Tangl [100]	Van Slyke and Bosworth [117]
	per cent	per cent	per cent	per cent
Carbon	52.96	54.00	52.69	53.50
Hydrogen	7.05	7.04	6.76	7.13
Nitrogen	15.85	15.60	15.65	15.80
Sulfur	0.72	0.77	0.83	0.72
Phosphorus	0.85	0.85	0.88	0.71
Oxygen (by difference)	22.77	21.74	23.19	22.14

proved to be unwarranted, although the different species appear to have practically the same compositions. However, the gross elementary composition is constant and there is probably uniformity in the proportion of structural units.

Amino acid content. Since the more conventional methods of organic chemistry have yielded very little information of value regarding the structure of proteins, the procedure of acid hydrolysis has been extensively used in an effort to obtain knowledge of the character and relative proportions of the components of this class of substances. Table XLI is a compilation of the more important results obtained by the attempts at isolation of the amino acids of casein. Results for cystine obtained colorimetrically are also included. It should be borne in mind that higher figures are usually closer to true values, since it is rarely possible to approach complete recovery. Because of the involvement of water in the hydrolysis, the sum of the amino acids, ammonia, sulfur and phosphorus should be somewhat greater than 110 per cent of the weight of the dry casein. The values so far obtained give a total of less than 106

Table XLI.—Amino acid components of casein.

	per cent	per cent	per cent	per cent
Glycine (α-amino acetic acid)	0.00 [81]	0.45 [39]	......	......
Alanine (α-amino propionic acid)	1.50 [81]	1.85 [39]	......	......
Valine (α-amino isovaleric acid)	7.20 [81]	7.93 [39]	......	......
Norvaline (α-amino valeric acid)*	0.2 [7]	......	......	......
Leucine (α-amino isocaproic acid)	10.5 [1]	9.70 [39]	7.92 [63]	9.35 [114]
Isoleucine (β-methyl α-amino valeric acid)	......	......	1.43 [63]	......
Phenylalanine (β-phenyl α-amino propionic acid)	3.2 [1]	3.88 [39]	......	......
Tyrosine (β-parahydroxyphenyl α-amino propionic acid)	4.5 [1]	5.70 [14]	7.49 [59]	......
Serine (β-hydroxy α-amino propionic acid)†	0.43 [35]	0.40 [39]	0.8 [88]	......
——— (hydroxy α-amino butyric acid)‡	......	......	1.8 [88]	......
Proline (α-pyrrolidine carboxylic acid)	......	7.63 [39]	8.7 [28]	6.70 [114]
Hydroxyproline (γ-hydroxy α-pyrrolidine carboxylic acid)	0.23 [35]	......	......	......
Glutamic acid (α-amino glutaric acid)	15.55 [81]	21.77 [39]	21.6 [28]	......
Hydroxyglutamic acid (β-hydroxy α-amino glutaric acid)	......	......	10.5 [28]	......
Aspartic acid (α-amino succinic acid)	1.39 [81]	1.77 [39]	4.1 [28]	......
Tryptophane (β-indol α-amino propionic acid)	1.5 [1]	2.23 [59]	1.7 [28]	......
Arginine (δ-guanidine α-amino valeric acid)	4.84 [51]	3.81 [80]	5.81 [59]	5.2 [40]
Histidine (β-imidazole α-amino propionic acid)	3.39 [35]	2.50 [80]	3.64 [59]	......
Lysine (α-ε-diamino caproic acid)	......	5.95 [80]	5.18 [59]	......
Cystine (α-α′-amino propionic acid β-β′-disulfide)	0.25 [37]	0.34 [59]	0.21 [121]	......
Methionine (γ-methylthiol α-amino butyric acid)	0.40 [74]	......	......	......
An amino acid, $C_{12}H_{26}N_2O_5$	0.75 [36]	......	......	......
Ammonia	1.60 [79]	......	......	......
Other amino acids reported "present"	[5, 29, 38]	......	......	......
Total of highest values, including 2.6 per cent H_3PO_4 and 0.7 per cent sulfur		105.65		

* Probably more than 1.0 per cent. See ref. 7.
† The value 0.8 per cent probably represents about one-half the serine. See ref. 88.
‡ The value 1.8 per cent probably represents about one-half of this acid. See ref. 88.

per cent. Values from colorimetric methods of determination of certain of the amino acids indicate that these acids are present in casein in higher percentages than those indicated by isolation methods. Furthermore, there is considerable positive evidence proving the presence of undetermined components in casein.

Nitrogen distribution. A somewhat different method of determining the character and amount of amino acids in a protein is the Van Slyke scheme.[116] Table XLII shows the nitrogen distribution of casein as determined by this method by several investigators. Comparisons of the values of Table XLII may be made with those of Table XLI only in the cases of cystine, arginine, histidine and lysine. When these are calculated to the same basis, there is substantial agreement except in the case of cystine for which the data are somewhat variable. The ammonia obtained probably results from splitting of acid amide linkages during hydrolysis.

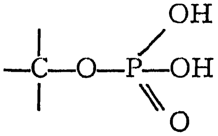
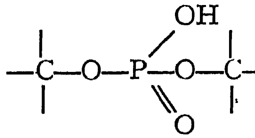
Table XLII.—Nitrogen distribution of casein.

	Van Slyke [116]	Crowther and Raistrick [22]	Dunn and Lewis [31]	Hoffman and Gortner [56]	Jones and Gersdorff [59]
	per cent	per cent	per cent	per cent	per cent
Ammonia nitrogen...	10.27	10.25	10.49	10.20	11.53
Humin nitrogen	1.28	1.20	2.13	1.51	1.27
Cystine nitrogen	0.20	1.24	0.48	1.05	0.89
Arginine nitrogen ..	7.41	9.22	7.42	9.20	11.60
Histidine nitrogen ..	6.21	6.82	6.01	6.26	6.12
Lysine nitrogen	10.31	9.62	9.09	8.49	6.17
Amino nitrogen in filtrate from bases...	55.81	54.76	58.78	54.12	59.77
Non-amino nitrogen in filtrate from bases	7.13	7.09	5.93	8.76	2.63
Total	98.61	100.19	100.33	99.59	99.98

Sulfur and phosphorus. The sulfur of casein has been in part accounted for by Mueller [74] as present in a sulfur-containing amino acid, later identified and named methionine by Barger and Coyne,[9] and in part identified as present in cystine as a disulfide linkage. The major part is still unaccounted for. Jones and Gersdorff [59] have shown that repeated precipitation of casein from solutions in dilute sodium hydroxide considerably reduces the percentage of cystine in casein.

The evidence has indicated that the organically combined phosphorus is present in casein as phosphoric acid groups. Recently, the composition of the polypeptide groups with which the phosphoric acid is associated has been investigated and evidence has appeared which, although conflicting in some details, is of real value in interpreting results reported on fractionation of casein and on its molecular weight.

Posternak [86] has reported the isolation from casein, after tryptic digestion, of several polypeptides, each containing over 5 per cent of phosphorus. It is difficult to evaluate his work because of paucity of details.

Rimington,[88] using the same method of digestion, isolated a pure phosphorus-containing polypeptide, "phosphopeptone," of an ultimate composition corresponding to $C_{37}H_{62}O_{33}N_9P_3$. The amount obtained corresponded to approximately 50 per cent of the phosphorus in the digest and contained 7.05 per cent phosphorus. From phosphopeptone after hydrolysis were isolated hydroxyglutamic acid, hydroxyaminobutyric acid and serine, but no other amino acids. The molecular proportion of these acids was 3, 4 and 2, respectively, to 3 of phosphoric acid. Since only two-thirds of the total phosphorus in phosphopeptone is removed by bone phosphatase, and the remaining one-third by kidney esterase, Rimington concludes that two-thirds is in the linkage  and one-third in the linkage  two amino acid groups being involved in this latter case. It appeared further to be probable that the remainder of the phosphorus in casein is similarly combined.

Since the percentage of phosphorus in phosphopeptone is practically 10 times that in casein, and since phosphopeptone contains only half of the phosphorus of the original casein, half of the total phosphorus of casein must be segregated in not more than one-twentieth of the polypeptide components of casein, and it seems probable that all of the phosphorus is segregated in not more than one-tenth of the polypeptide groups. Since it is possible to fractionate casein into high-phosphorus and low-phosphorus portions (see following paragraphs), it appears that phosphopeptone (and consequently phosphorus) either is present only in certain species of casein molecules or else is present in much higher proportions in some molecules than in others.

Identity. It was noted earlier that no significant differences have been detected among the elementary compositions of caseins from the milks of different mammals. The same striking agreement has been observed among the highly delicate immunological reactions,[127] the amino acid contents [2] and the spectrophotometric characteristics.[6] A comparison of the optical activities of the amino acids of racemized casein (q.v.) from cow's milk and sheep's milk by Dudley and Woodman [80] has led to the conclusion that, although the proportions of the components in caseins from different sources are probably the same, the structural arrangement of the amino acid units varies with the species of the source.

Heterogeneity and molecular weights. Because in so many ways casein acts like a single substance, it was quite generally assumed, until after 1920, that acid-precipitated casein was a single chemical entity of

definite composition and molecular weight. Work at the Carlsberg laboratories [60, 69] showed, however, that the solubility of casein in dilute hydrochloric acid is a function of the amount of casein present, this evidence being taken to prove that casein is a mixture of two or more proteins, presumably similar in chemical composition, which unite in varying proportions to form complexes differing in solubility. This work was successfully extended to fractionation,[68, 69] the extreme fractions differing appreciably in solubility and phosphorus-nitrogen ratio.

Subsequently, Linderstrøm-Lang [70] carried out successive fractionations of carefully prepared casein, using acidified alcohol as the differentiating solvent. He found the fractions to differ considerably in analytical composition, chemical activity and physical characteristics. The fractions recombined in the proper proportion resembled the original casein in respect to all properties compared, this indicating that the separation had been a merely physical one. Rate of digestion of trypsin-kinase, ratio of formol titratable nitrogen to total nitrogen, fractionation by rennin action and by calcium chloride solution, were some of the characteristics employed in the comparisons.

Cherbuliez and coworkers [18] have fractionated casein, using ammonium chloride solutions as the means, and have obtained fractions differing most remarkably in phosphorus percentages,—0.72 to 2.32. By control of the reaction and use of acetone as precipitant, this method of fractionation yielded four homogeneous components which differed particularly in tryptophane content and coagulability by rennet. Supplee [105] has obtained similar fractionation in respect to phosphorus percentages by successive sedimentation of finely precipitated casein. The previously discussed work of Rimington [88] on the manner in which phosphorus is combined in casein is of interest here in suggesting details of the course of fractionation.

The molecular weight of casein was first directly determined by Svedberg, Carpenter and Carpenter [108] by means of the centrifugal sedimentation velocity method. They found casein to be a mixture of proteins, the larger proportion of the substance having a molecular weight of between 75,000 and 100,000, and the smaller proportion, extracted by hot acidified ethanol, having a molecular weight of 375,000 ± 11,000. Crude casein subjected to a temperature of 40° during the time of dissolving in buffer solutions was found to have associated or polymerized to molecules of 188,000 molecular weight. This latter fact appears to justify the doubt as to whether the value of 375,000 really represents the molecular weight of a substance present in casein before the treatment with hot alcohol. Existing evidence seems insufficient to settle this question.

Carpenter [17] has analyzed the casein fraction having a molecular weight between 75,000 and 100,000 as shown by sedimentation, determining sulfur, phosphorus, cystine, tryptophane, tyrosine and histidine. By application to these values of the principle of the common multiple, he found the most probable value for the molecular weight of this fraction

to be 98,000. This molecular weight corresponds to a formula of the order of $C_{4400}H_{7000}N_{1100}O_{1400}S_{22}P_{22}$.

By means of serological reactions, Carpenter and Hucker [16] found the three proteins of molecular weights 98,000, 188,000 and 375,000 to be readily distinguishable from one another. They also obtained serological evidence that the alcohol-soluble protein of Osborne and Wakeman [83] is not identical with the acid-alcohol-soluble protein of molecular weight 375,000.

General properties. The casein of commerce usually appears as a yellowish-white powder which is quite stable if reasonably dry, but, if moist, undergoes putrefactive decomposition. Refined casein is a white powder which is not appreciably hygroscopic. It is not readily wetted by water or aqueous solutions unless it already contains traces of water, but floats on the surface. It is insoluble in water, alcohols and most neutral organic solvents. Propionic and acetic acids by themselves have no solvent action on casein, but in the presence of certain amino acids or benzene derivatives they acquire solvent power. Formic, lactic and pyruvic acids dissolve casein and a lactic acid solution may be diluted with ethanol, or a pyruvic acid solution with acetone, without precipitation. These solutions in the mixed solvents may be further diluted with water without becoming clouded. Casein is insoluble in dry pyridine, but addition of water causes solution. Casein, if previously wetted with a small quantity of the solvent, dissolves readily in solutions of the alkalies or of the common alkaline salts. It is insoluble in cold, dilute, inorganic acids, but on warming in contact with acid solutions of hydrogen-ion concentration greater than indicated by pH 2.5, hydrolysis takes place. Casein may be dispersed in acid solutions, but it is preferable to use a weak acid in order to avoid a hydrogen-ion concentration that might cause hydrolysis. The specific gravity of casein lies between 1.25 and 1.31. Michaelis and Pechstein [72] report the isoelectric point of casein to be at pH 4.6. The specific rotation of casein dispersed in dilute orthophosphoric acid or in acetic acid has been reported by Rakuzin [87] as $[\alpha]_D = -86.6°$. He states also that, if the solvent is a 2 per cent solution of borax or a 2 per cent hydrochloric acid solution containing 0.2 per cent pepsin, $[\alpha]_D$ lies between $-93.5°$ and $-95.4°$. Zaykowsky [131] reported $[\alpha]_D^{18°} = -83.59°$ for 2 per cent pure casein dissolved in a 10 per cent sodium acetate solution. Gould,[43] using Zaykowsky's method, found $[\alpha]_D^{20°} = -81.7°$ for Hammarsten casein.

Coagulation. Casein may be precipitated from its dispersions by bringing its environment close to the isoelectric point of casein, by the action of rennet, by heating, by addition of ethanol, and by addition of salts. This topic will be discussed in detail in later chapters.

Combining capacity. Casein, like other proteins, is amphoteric,—that is, it combines with either acids or bases. Bancroft and Barnett [8] have found that casein will combine directly with HCl gas, but not with NH_3. In equilibrium with an aqueous environment at its isoelectric point, casein is considered to be uncombined; at greater hydrogen-ion

concentrations, it is combined to some extent with acid; at lower hydrogen-ion concentrations (greater hydroxyl-ion concentrations), it is combined to some extent with base. Cohn and Berggren,[20] using the potentiometric method, studied the base-combining capacities of ten casein preparations and found that these differed from one another to a greater extent than could be accounted for by experimental errors involved in the measurements and calculations. The variations were systematic and could be explained as dependent upon the method of preparation of the casein. Casein that had not been exposed to greater alkalinities than that of milk combined with approximately 0.0014 mols of NaOH per gram at 21°; casein prepared "nach Hammarsten" and casein that was saturated with base during its preparation combined with approximately 0.0018 mols of NaOH per gram. One mol of NaOH, therefore, combined with 735 grams of casein that had not previously been exposed to alkaline reactions, or with 535 grams of casein that had previously been saturated with base. Much less alkali is bound by casein at 35° than at 25°, and less at 25° than at 15°.

In these experiments, both the amount of casein used and the concentration of NaOH were varied, and it was found that in every preparation the amount of base bound by the protein was independent of the concentration of the free base. Accordingly, these authors conclude that this neutralization is chemical in nature, being dependent on the dissociation of the acid groups in the casein molecule.

Racemized casein. If a protein solution is treated with dilute alkali and the mixture incubated at 37° or at room temperature, the optical rotation of the solution steadily decreases, approaching a practically constant value. Hydrolysis of the product produces amino acids, some of which have completely lost their optical activity, others of which retain their original activity. Dakin [25] believes that the cause of this phenomenon lies in a keto-enol tautomeric change involving a hydrogen on an

$$-\overset{\text{H}}{\text{N}}-\underset{\text{R}}{\overset{\text{H}}{\text{C}}}-\text{CO}-\overset{\text{H}}{\text{N}}- \rightarrow -\overset{\text{H}}{\text{N}}-\underset{\text{R}}{\text{C}}=\text{C(OH)}-\overset{\text{H}}{\text{N}}-$$

a carbon atom and the adjacent carbonyl of the peptide linkage. The asymmetric carbon atom thereby loses its optical activity and, furthermore, when the double bond is ruptured by subsequent hydrolysis, there will result a racemic mixture of the dextro and levo forms of the amino acids involved. Consequently the amino acids will be optically inactive. He believes that, in order for racemization to take place, it is necessary that other groups be attached to both the amino and the carboxyl radicals and that, lacking such groups, the terminal groups of the peptide chains are not racemized, hence the apparently selective character of the racemization process. This selectivity has been made use of in determining the identity of caseins from milks of various animals, as mentioned earlier in this chapter.

This theory of Dakin's has been attacked on the grounds of failure

to find spectrographic evidence of existence of the double bonds required by enolization. Gröh and Weltner [44] have made spectrographic studies on a number of proteins in alkaline solution and found that the enolization of some proteins takes place slowly and the subsequent change to the inactive keto form rapidly, in which case the desired spectrographic evidence is practically entirely lacking. They found that other proteins yield a more stable and relatively permanent enol form and give positive evidence of the existence of the double bond linkage. The Dakin theory is, therefore, to be considered reasonable, at least.

From the facts that the yield of racemized casein is only about 20 per cent of the original casein, that considerable ammonia is evolved during the process, and that racemized proteoses remain in the mother liquors after the precipitation of the racemized casein, Dakin and Dudley [26] concluded that, in the case of casein, considerable hydrolysis takes place during the racemization process. This might account for the variable results obtained by Cohn and Berggren [20] in determining the combining capacity of casein. Dakin and Dudley [27] also found that racemized casein was not digested by pepsin, trypsin, or erepsin, *in vitro,* nor was it digested by dogs. It was not affected by putrefactive bacteria during a 10-day exposure.

Combinations of halogens with casein. In Table XLIII are listed the percentages of the various halogens that have been introduced into the casein molecule. Combinations containing lesser percentages each of halogens have been produced by treating the higher percentage combinations with water, with ethanol, or with ether, or by dissolving them in dilute alkali and precipitating with acid.

Table XLIII.—Halogen compounds of casein.

Investigator	Halogen introduced	Halogen found
		per cent
Habermann and Ehrenfeld [45]	Chlorine	13.28-14.04
Panzer [86]	Chlorine	8.32
Salkowski [92]	Chlorine	6.72-6.78
Vandevelde [113]	Chlorine	32.5
Hopkins and Pinkus [57]	Bromine	11.17
Vandevelde [112]	Bromine	32.3-35.2
Lepinois [62]	Iodine	21.6
Liebrecht [67]	Iodine	17.8
Masui [71]	Iodine	10.81

Desaminocasein. This compound, prepared by the action of nitrous acid on casein, has been studied by Skraup and Hoernes,[100] Levites,[64, 65] Dunn and Lewis,[31] Lewis and Updegraff,[66] and Steudel and Schumann.[102] From 90 to 97 per cent of the weight of the original casein may be obtained by means of the nitrous acid treatment as a yellow powder which gives a red solution in dilute NaOH solution. Practically all of the ϵ-amino group of the lysine is removed from the casein in the process and the percentages of several of the other amino acids are apparently

somewhat reduced. Desaminocasein lacks the nutritional adequacy characteristic of casein,[103] evidently due to the loss of the lysine grouping.

Nitrocasein. Von Furth[122] nitrated casein at room temperature, adding urea to eliminate the effect of nitrous acid on the reaction. He obtained a bright yellow powder, soluble to a red brown solution in alkalies, and precipitated therefrom by acetic acid. It gave neither Millon's nor the lead acetate reaction. A slightly different product was obtained by nitration in the absence of urea.

Methylated casein. Skraup and Krause[101] treated casein with methyl iodide and KOH in alcoholic solution and obtained a compound which contained, not only an increased percentage of methoxy and methylamino groups, but also a considerable proportion of iodine. Geake and Nierenstein,[42] using diazomethane as a methylating agent, increased the methyl groups in casein by about 3 per cent. Their product gave all the color reactions characteristic of casein. Herzig and Landsteiner[61] by the same method succeeded in increasing the percentage of methyl groups by more than 6 per cent. Edlbacher[32] used dimethyl sulfate with apparent success.

"Protein poison" from casein. As first shown by the Vaughns,[120] when proteins are hydrolyzed by heating in a 2 per cent solution of NaOH in absolute ethanol, a product is obtained which may be divided into two fractions, one of which is insoluble in absolute ethanol and is physiologically inert, the other of which is soluble in absolute ethanol and is extremely poisonous when injected intravenously, intraperitoneally, or subcutaneously into animals. The symptoms produced are similar to those of anaphylaxis. Such a substance has been prepared from casein and its properties investigated, but its exact composition is not known.

Formaldehyde casein. A plastic product of considerable commercial importance is made from casein. Formaldehyde is used to harden these plastics, but little is known regarding the fundamental chemistry involved in the hardening reaction. Benedicenti[12] prepared a combination of casein and formaldehyde and found that all of the formaldehyde could be recovered from it by steam distillation and that the casein was apparently unchanged except for slight hydrolysis. Formaldehyde casein will be mentioned later in this chapter in connection with the uses of casein.

Carbon disulfide casein. Carbon disulfide reacts with casein in the presence of alkali. Uhl[111] believes that the reaction is

$$R\text{—}NH_2 + CS_2 \rightarrow R\text{—}NH\text{—}\overset{\|}{C}\text{—}SH$$

He prepared what he believed to be a copper salt of this compound. However, he used concentrations of alkali amply sufficient to hydrolyze the casein and the substance he obtained very probably consisted of amino thioacids. Gould (unpublished work), using a minimum quantity of alkali, obtained a reaction which produced large amounts of H_2S. He

obtained from the solution by precipitation with acid a substance having the same percentage sulfur as the original casein.

Paracasein. Casein is converted by the action of the enzyme rennin into paracasein, a substance differing from casein in several properties. Elementary analyses and nitrogen distribution values [41, 117] reveal no significant changes in composition. It is generally believed that the difference is a physico-chemical one, although no entirely adequate theory has yet been advanced. Paracasein will be discussed in more detail in the chapter dealing with rennet coagulation.

Compounds of casein. Numerous attempts have been made to obtain compounds of casein with metals, salts, organic substances, etc. Among the compounds reported to have been prepared are those with the alkali metals, alkaline earths, copper, cobalt, mercury, silver, iron, arsenic, phosphates, alums and alkaloids. Certain of these have been found to be of medicinal value and will be mentioned in that connection later.

Decomposition products. A discussion of decomposition products of casein would necessitate extensive discussion of many topics of minor importance from the standpoint of milk and milk products. Certain of these products of oxidation and of partial hydrolysis of casein are treated by Gortner in Sutermeister's monograph on casein.[106]

Preparation of Casein

Preparation of casein for research purposes. Casein "nach Hammarsten" is prepared by the following method.[49] Skim milk is diluted with four times its volume of water and the casein precipitated by the addition of dilute acetic acid. The casein is then purified by repeatedly dissolving in water containing the least amount of alkali possible, filtering, reprecipitating with dilute acetic acid, and washing with water. The casein must not be exposed to high concentrations of hydroxyl ions on account of the danger of racemization; it must not be exposed to high concentrations of hydrogen ions on account of the danger of hydrolysis. Without previous drying, it is triturated with absolute ethanol, the alcohol sucked off, the casein treated with anhydrous ether, the ether sucked off, and the product dried either over H_2SO_4 at reduced pressure or over $CaCl_2$ at atmospheric pressure. The first method of drying gives a product that is difficult to wet with water, the second method one that contains traces of water and is therefore readily wetted.

Robertson [89, 90] claims that casein prepared by the Hammarsten method contains a small amount of a water-soluble, acid substance, which he removes by the following treatment. A kilo of the casein is triturated with 2 liters of distilled water, the casein allowed to settle and the supernatant water decanted. This is repeated until 12 kilos of water have been used. The casein is then washed with successive small portions of 5 kilos of absolute ethanol, and then with successive small portions of 5 kilos of ether which has previously been distilled over sodium. After the last washing, the casein is drained as free from ether as possible without

allowing opportunity for condensation of water on the product and placed in a desiccator over H_2SO_4 at 40 to 50° and the source of heat removed. At the end of 24 hours, the casein is spread out in a thin layer in the desiccator and the temperature raised to 40-50°. At the end of another 24 hours, the substance should be a white, pure product, free from water and from ether. To wet it, it is necessary first to rub it to a paste with a small amount of the solvent.

Van Slyke and Bosworth [117] state that Hammarsten casein contains about 0.85 per cent of phosphorus, which is 0.14 per cent too high for pure casein if the assumption is made that the molecule contains equal numbers of phosphorus and sulfur atoms. They attribute the excess of phosphorus to the presence of dicalcium phosphate and describe a method whereby they removed this phosphate and obtained a casein containing only 0.71 per cent phosphorus.

Separator skim milk is diluted with seven or eight times its volume of distilled water and a 6 per cent solution of acetic acid is added slowly until the casein is completely precipitated. As soon as the precipitate settles, the clear supernatant liquid is syphoned off. The casein is washed several times with distilled water by decantation and then dissolved in dilute NH_4OH. Six cc. of concentrated ammonia solution are diluted to a liter and this quantity of reagent used for each liter of milk originally taken. The solution is filtered through absorbent cotton and the whole procedure of precipitating, washing, redissolving and filtering repeated at least four times. Finally an excess of concentrated NH_4OH solution is added, followed by saturated ammonium oxalate solution. The liquid is centrifuged and filtered, and the casein precipitated from the filtrate by dilute HCl (10 cc. HCl, sp.g. 1.20, per liter). After being washed till free from chlorides, the casein is filtered on a Büchner funnel and treated with absolute alcohol and ether as previously described.

More recently these same investigators [118, 119] have described a somewhat different method which they claim gives a casein free from inorganic phosphorus, calcium and hydrolytic products. Fresh undiluted milk is treated with $N/1$ acid, either lactic or a mixture of HCl and acetic, the acid being delivered under the surface of the milk and very close to a mechanical stirrer which rotates at a rate of about 2000 R.P.M. The acid is added in several portions, the mixture being stirred slowly for several hours between additions. After the casein is all precipitated, the liquid is removed by centrifuging and the casein washed by successive stirring with water and centrifuging four or five times. Tenth normal alkali is then added to the casein suspended in water until the reaction is at pH 7.0. Calcium and magnesium phosphates are then largely removed by centrifuging. The casein is next precipitated by means of $N/1$ acetic acid, the reaction being brought to pH 4.7. The solid is centrifuged out, resuspended in water and the reaction adjusted to pH 4.7. The suspension is then electrolyzed in a three-compartment cell to remove traces of phosphates, and finally filtered and treated with anhydrous solvents to remove water.

The above method has been described in a modified form by Cohn and Hendry,[21] who offer no analytical evidence to indicate any points of superiority of their product. Their principal modification is to dissolve the casein in alkali, the reaction being at pH 6.3 (colorimetrically determined) when reaction is complete. By filtration at this reaction they claim to remove calcium caseinate as well as calcium phosphate and fat.

Precipitation of casein at pH 4.1, as in the commercial grain-curd process described below, followed by thorough washing, will remove all but about 0.20 per cent calcium oxide and all of the phosphorus pentoxide in excess of 1.80 per cent. For most purposes the small quantity of extraneous ash present—less than 0.50 per cent—is not objectionable and, a matter of considerable importance, the casein will not have been exposed to alkalinities greater than that of milk itself.

Preparation of casein for commercial use. Commercial casein is made by either of two general methods,—precipitation by acid or coagulation by rennet. The rennet method is used almost entirely and exclusively for producing casein for plastics manufacture, since rennet casein has the peculiar properties considered essential for this product,—properties apparently closely related to its high ash content. Acid precipitation is employed for producing casein for its other commercial uses, all of which depend on its adhesive properties.

In the manufacture of rennet casein,[75, 101] a fresh, low-fat, skim milk is warmed to 96° F. (35.5° C.) and curdled with about 4 ounces of rennet to every 100 gallons of milk. The curdling should be complete in 15 to 20 minutes, after which the curd is broken up and gradually heated to 130 to 150° F. (55 to 65° C.). After settling for 10 minutes, the curd is drained of whey and washed several times with water at 80 to 90° F. (26 to 32° C.). It is then pressed for an hour, shredded, and dried in thin layers at 110 to 115° F. (43 to 46° C.). Rapid drying at a low temperature is essential if the product is to be of a light color.

Acidification methods may be of the self-sour type, in which the acid is formed in the skim milk by bacterial fermentation of lactose to lactic acid, or of the type in which acids are added to the milk in sufficient quantity to precipitate the casein and attain the desired degree of acidity. There are numerous modifications depending on the acids and the temperatures used and on the mechanical arrangements. Equipment has recently been devised for continuous precipitation, washing and drying of casein, which, as might be expected, gives a product of remarkable uniformity.

In the self-sour process,[24] the milk is inoculated with lactic acid bacteria and allowed to stand at a favorable temperature until curdling takes place. The curd at this acidity,—about pH 4.7,—is soft and fine, and, in order to agglomerate it, heat is used. Too high temperatures produce a rubbery curd that is impossible to wash successfully unless it is chopped by mechanical means. After draining, the curd is washed several times, drained, pressed over night, shredded, and dried at 130° F. (54° C.). This type of casein, unless exposed to greater acidity than pH 4.7, will

contain a somewhat greater percentage of ash than the grain-curd type next to be described.

If any dilute strong mineral acid, such as hydrochloric or sulfuric, be added to skim milk at 95° F. (35° C.) in sufficient quantity to bring the reaction to pH 4.1 (apparently pH 4.6, if methyl red is used as indicator), a curd of coarse, granular texture is formed.[19] This is known as grain-curd and, because of its open texture and of the fact that calcium phosphate is completely in solution at this acidity, is easily washed to produce a low-ash, low-acid casein. If the casein is to be used for coating paper, it is desirable that the agitation be moderate and the temperature be not below 95° F. during precipitation in order to avoid tendency of the casein to cause foaming in the coating slip. Higher temperatures may be used, provided the curd is thoroughly chopped before washing. The washing, pressing, shredding, and drying operations are the same for all types of curd. Casein for paper coating should be ground to pass a No. 30 screen, the proportion of finer particles being kept as small as possible.

Casein is made from buttermilk to some extent, but the color is usually darker and the quality in general usually poorer than that of casein made from skim milk. Ranges of composition of typical commercial caseins will be found in Chapter I.

Uses of Casein

Paper coating. The most extensive use of casein in the United States is in the coated paper industry where it serves as an adhesive to bind such minerals as china clay, satin white, and coloring pigments to the surface of the paper so as to make them an integral part of the paper. Other adhesives used for this purpose, notably animal glue and starch, have been largely supplanted by casein because it makes possible a finish of higher gloss and superior printing properties. Such properties are particularly valuable for coating paper for fine lithographic work. Casein bound coatings can be made highly resistant to water, by means of formaldehyde or other "insolubilizer," a process that is useful in making wall paper, playing cards and other washable papers.

The casein for paper coating is first softened with water, then dissolved in the alkaline solvent, and this solution mixed thoroughly with a water suspension of the pigments. The mixture is then diluted with water to give the desired consistency and applied to the paper either by rotating brushes or by rollers.

The solvents commonly used are solutions of sodium carbonate, borax or ammonia, and less commonly caustic soda or trisodium phosphate, these alkalies being used either in mixtures or separately. The quantity of casein required depends on its adhesiveness, the finish desired and the pigment employed, the ratio of casein to dry pigment ranging from 15 to 25 per cent.

The characteristics chiefly desired in a casein for paper coating are maximum adhesiveness, freedom from foreign matter, minimum ash and

acidity, good solubility, and freedom from tendency to cause foaming during the coating process. The essentials in a process for making a casein to meet these requirements are moderate agitation and a temperature not lower than 95° F. (35° C.) during precipitation, acidification to pH 4.1, thorough washing of the curd, drying at a temperature not above 130° F. (54° C.), and scrupulous cleanliness throughout the process.

Casein is also employed in the paper industry to some extent as a dispersing agent for the rosin used for sizing paper.

Glues. Casein glue first became familiar to the woodworking industries of this country during the World War, when it was used extensively for certain purposes in airplane construction, especially for plywood for fuselage covering, engine beds, etc. The experience so obtained revealed its many advantages and led to its widespread adoption for certain peacetime applications.

Casein glue was originally introduced because, unlike the glues which it replaced, it is resistant to water. Properly prepared wood joints made with casein glue will withstand soaking in water for several days without coming apart and, on heating to high temperatures, they will retain their strength better than similar joints made with other glues. The use of casein glue at the present time is often due to certain advantages other than water resistance. The glue is marketed in the convenient form of a dry powder containing besides the casein such substances as hydrated lime and sodium carbonate and possibly other compounds. The glue can be prepared within a few minutes by dissolving in water without the necessity of heating the mixture, is applied cold, and requires only presses of the types commonly used in woodworking. Casein glues have only a short working life and therefore must be made up not more than a few hours previous to use.

Casein glue is used in such woodworking industries as the making of automobile body frames, pianos, furniture, doors, millwork, refrigerators, and in general uses where a water resistant glue is desired. It is especially useful for fastening surface material to the running boards of automobiles. Special glues or pastes are used for fastening labels to cans and glass containers, insuring adhesion under the most trying conditions.

Plastics. The manufacture of plastics from casein is a comparatively new industry, commercial production having started since the beginning of the twentieth century. In 1930, the world's annual production was approximately 10,000 tons.[98] Although they are marketed under numerous trade names, the term Galalith is quite generally used to connote casein plastics, whatever their source.

The dry process,[98] which is the only process now in commercial use, employs a rennet casein low in fat, high in ash and moisture, and free from dirt, specks and splinters. The earlier wet process, which used acid-precipitated casein, has never been a commercial success, although it is true that a small proportion of acid casein is sometimes mixed with rennet casein for plastics.

The rennet casein, ground to pass a No. 40 screen but not a No. 80, is mixed with water up to approximately 40 per cent of its net weight and the mass stirred in a dough mixer. Coloring materials are added in the mixer, pigments being avoided as much as possible, fast acid dyes being chiefly used. The thoroughly mixed material is extruded through a nozzle at a carefully controlled temperature. If sheets are desired, the extruded rods are pressed in heavy multiple platen presses, hydraulically operated. The shaped material is soaked in formaldehyde solution of a concentration and for a time depending on the thickness of the pieces. The product is then dried and marketed.

Casein plastics are used as substitutes for horn, celluloid, bone, ivory, ebony, pearl, amber, jade, lapis lazuli and tortoise shell. The material is relatively non-inflammable, it is cheap, it can be turned or drilled, it has a high tensile strength, it can be produced in a great variety of colors and can be made transparent, translucent or opaque. On the other hand, it has certain disadvantages, nearly all being related to its absorption of water and concomitant swelling. Friction joints in fountain pens stick in damp weather, articles frequently exposed to water develop fine cracks and warp. Plastic casein is a satisfactory electrical insulator as long as it is dry, but loses this property almost completely when wet.

Plastic casein is most often seen in the United States in the form of fancy buttons, but it is also used for such articles as umbrella handles, costume jewelry, toilet sets, cigarette holders and spectacle frames.

Paints. The use of casein as a binder in cold water paints for both interior and exterior application, especially where a comparatively inexpensive coating is desired, is increasing considerably. Casein is readily dissolved by cold solutions of alkalies such as sodium carbonate, caustic soda, trisodium phosphate and borax; if the mixture also contains lime, aldehyde ammonia or hexamethylene tetramine, the casein subsequently becomes insoluble, so that the dried paint is resistant to moisture. The pigments commonly used in casein paints are china clay, barytes, whiting and magnesia for whites, and ferric oxide, carbon black, chromates, ultramarine and Prussian blue for colors.

Lime is the most commonly used insolubilizer on account of its comparative cheapness, but the proportion used must be very carefully regulated. An excess of lime hinders solution of the casein and causes the dried paint to flake off; too little lime will result in the paint being somewhat soluble. The insolubilizing action of formaldehyde may be employed if it is used in its combination with ammonia as hexamethylene tetramine in order to make the action slower. Its comparatively high cost prevents its use to any large extent. Aldehyde ammonia is used to obtain the insolubilizing action of acetaldehyde, the ammonia in combination acting to retard the reaction.

In making cold water paint with casein as binder, the powdered casein, hydrated lime and other alkalies, pigments and fillers in the proper proportion are mixed dry and packed in air-tight cans. The paint may then

be easily prepared immediately before use by mixing with the specified amount of water.

Although the greatest use of casein paints in the past has been for interior walls and ceilings, their modification by incorporation of drying oils, rubber latex, phosphates, asbestos, sodium silicate and plasticizers makes possible many other highly specialized uses.

Foods and medicinal preparations.[76] Casein, usually in the form of ammonium or sodium caseinate, is a constituent of many of the patented and medicinal foods on the market, particularly those recommended for infants, dyspeptics, diabetics and persons afflicted with tuberculosis. The percentage of casein may be as great as 95 per cent. A carefully made casein, odorless, tasteless and light in color, is used. Casein has been reported as an adulterant in certain foods in which its capacity to absorb and hold water is advantageous. Medicinally, casein is employed as an emulsifying agent for oils for internal administration, as a vehicle for the administration of lithium, mercury, arsenic, iron, silver and iodine, and in compounds of therapeutic value with salicylates, acrolein, hydrobromic acid and hydriodic acid.

Textiles and leathers.[94] Casein is used in the textile industry in printing calico, where it serves to fix the color by the reaction involved in the drying of the alkali casein combination. It is also used in combination with powdered mica, soluble salt and ammonia for printing cloth to give it a metallic luster simulating bronze powder; for softening, sizing, and loading textile fabrics; for treating yarns; for a waterproofing and softening dressing; and for loading silk. Many processes are patented and some of them are perhaps not being used commercially to any extent.

A transparent wrapping material similar in appearance to cellophane, but reported to contain more than 75 per cent casein, has been produced and placed on the market. Details of its manufacture are not available.

In the manufacture of leather, casein is used as a finish for light leathers such as sheepskins in order to give the exterior surface a gloss, an operation known as "seasoning." [97] The casein is applied to the leather as a solution in the minimum quantity of borax, ammonia, caustic soda or sodium carbonate. A glazing machine is sometimes employed to increase the gloss after the drying of the casein film.

Adhesive spreaders. One of the more recently developed uses of casein offering a potential outlet for a considerable quantity of this material is in the manufacture of spreader for use in connection with the application of orchard and garden sprays. The function of spreaders, as the name would imply, is to spread the spray in a uniform film over foliage or fruit to give as nearly complete coverage as possible of insecticide or fungicide, and also to make the coating adhere better.

Spreaders must have certain characteristics to permit of their being used with safety to plants; to some there are objections that entirely exclude them from certain spray combinations because of the injury to the fruit or foliage which would result from the reaction between the spreader and the other spray constituents. Casein spreader acts as a

stabilizer and is popular because of the following outstanding features: 1. Compatibility with practically all spray mixtures and combinations; 2. Convenience in use,—requires no heating; 3. Exceptional spreading and adhesive properties; 4. Ready availability; 5. Cheapness.

The method generally used in preparing a spray necessitates having a product that dissolves quickly and without difficulty. This requires the use of good quality casein of the proper fineness uniformly mixed with the other ingredients. Some users prepare their casein spreader themselves, but the majority prefer to purchase a standard brand of prepared product because of its advantages from the standpoint of being more convenient to use and more uniform in quality. A satisfactory casein spreader may be made from a mixture of high purity hydrated lime and 60 to 80 mesh casein in a proportion of 2½ to 3 parts by weight of the lime to one of the casein, but other ingredients which are claimed to have certain beneficial effects are added by some of the manufacturers of the prepared spreader. The casein, lime and other ingredients are screened to remove lumps which might clog a spray nozzle and thoroughly mixed in a suitable mixer.

Miscellaneous uses. According to Scherer,[94] casein is used in the manufacture of asbestos paper and board to bind the material together; in shoe creams and polishes to give more gloss and shine to the finish; in making wood cement pulp roofing that is water and fire proof; in a mixture for coating the interior of barrels to produce a strong and water proof lining; in preparations for priming and preparing artists' canvas to receive the paint and to keep the canvas in shape; in a drying ointment with glycerine and dilute ammonia; for clarifying glues; in the manufacture of soap for improving the lathering properties and body; in making paint remover; in combination with formaldehyde for solidifying mineral oils; and in the making of face creams.

Casein is used as the binder in making composition cork for bottle cap disks and gaskets.[123] The casein is dissolved in water containing borax or other alkaline solvent to which glycerine has been added. An insolubilizer is added and the mixture is sprayed over ground cork which is then placed in iron molds and heated. After the molds have been cooled, the blocks of cork composition are removed, seasoned for a few days and cut into the form of sheets.

Proteins of Milk Other Than Casein

General discussion. After the casein of milk has been precipitated, there remain in solution two distinct proteins, lactalbumin and lactoglobulin. Lactalbumin is not identical with blood serum albumin, but is similar to it, as has been shown by various methods.[93, 120] Consequently, it appears that a distinct mammary synthesis is involved in the production of lactalbumin; that is, it apparently is not merely a transfer of albumin from the blood to the milk. The albumin of colostrum is identical with that of milk. Lactoglobulin, whether from milk or colostrum, is probably

identical with blood serum globulin.[22, 52, 93] Coagulation of these two proteins leaves in solution organic nitrogen corresponding to about 0.2 gram per liter of milk.[88] The properties and proportion of this residual protein vary considerably as found in the different investigations which have been carried out to define it more accurately, and it is quite probable that it is derived from the albumin or globulin, or both, during the heating required for their coagulation. Osborne and Wakeman[88] have obtained from milk an alcohol-soluble protein, which, from its very low phosphorus content, appears to be a distinct entity. From the nitrogenous film that surrounds the fat globules in milk has been isolated[4] a protein regarding which there has been much confusing evidence. Palmer and Wiese[84] have analyzed this film protein and found values for phosphorus, sulfur, nitrogen and nitrogen distribution which confirm the conclusion that this protein is not casein, lactalbumin, lactoglobulin or the alcohol-soluble protein of milk. They found it to be closely associated with phospholipids and to have its isoelectric point at pH 3.9 to 4.0. There has also been isolated from this film a mucoid protein, lactomucin[104] which on hydrolysis yields a substance capable of reducing Fehling's solution,—i.e., a carbohydrate (see Chapter III). The existence of a number of other proteins reported as occurring in milk is somewhat in doubt.

Preparation of lactalbumin. Lactalbumin may be prepared directly from milk by saturating with $MgSO_4$, filtering and adding 0.25 per cent acetic acid to the filtrate until it is permanently turbid. After standing for a time the precipitate is filtered out, redissolved, the solution neutralized, and the precipitation with $MgSO_4$ and acetic acid repeated from three to six times. It is sometimes recommended that the $MgSO_4$ solution be diluted with an equal volume of water before adding the acetic acid. Finally the residual $MgSO_4$ is removed by dialysis, the lactalbumin precipitated by ethanol, washed with ether, and dried at a low temperature.

Alcohol precipitation of lactalbumin from a practically salt-free solution gives a product readily soluble in water. Lactalbumin salted out—by $(NH_4)_2SO_4$ for example—is readily soluble in water, but alcohol precipitation from a solution containing salts in considerable quantity gives a relatively insoluble albumin. Heating to about 70° renders albumin practically insoluble, but coagulation is probably never complete at that temperature.

Coagulation and precipitation of lactalbumin. It has been found[91] in a series of experiments in which definite temperatures were maintained for 30 minutes that no lactalbumin was coagulated at 62.8°; 5.71 per cent was coagulated at 65.6°; 12.76 per cent was coagulated at 68.3°; and 30.87 per cent at 71.1°. Acidity aids the heat precipitation, the optimum for the mixed whey proteins being at pH 4.5.[77] Among other precipitants of lactalbumin may be listed saturated Na_2SO_4 solution, saturated $(NH_4)_2SO_4$ solution, tannin, and phosphotungstic acid. It is soluble in saturated NaCl and saturated neutral $MgSO_4$ solutions in contrast to casein which is insoluble in both. Acidification of a $MgSO_4$ solution precipitates lactalbumin. Rennet does not precipitate lactalbumin.

General physical and chemical properties. The statement that lactalbumin is altered by recrystallization is probably not valid,[95] but it has been proved that albumin crystals are not free albumin,[73] but rather a salt with the acid used in precipitation, e.g.,—acetate. All albumin crystals are crystallographically identical, or at least isomorphic.[128] They probably belong to the hexagonal system and are more or less positively doubly refractive. Lactalbumin shows various combinations of proto-prisms and proto-pyramids. In this form the crystals remain unchanged for a considerable time, but ultimately change from the monotropic α form into the enantiotropic β form and lose their optical properties. When quite dry they are stable at temperatures up to 150°.

The isoelectric point of lactalbumin is at pH 4.55 according to Csonka, Murphy and Jones,[23] and at pH 5.2 according to Sjögren and Svedberg.[90] The substance is levorotatory, but investigators do not agree on the magnitude of the specific rotation. It possesses a slight reducing power and forms an osazone,[61] which differentiates it from casein. A pure solution of lactalbumin gives a neutral reaction and the various characteristic tests of albuminous substances. Boiled with an alkaline solution of lead acetate, it gives a very strong sulfur reaction.

The molecular weight of lactalbumin has been determined by Sjögren and Svedberg who used the ultracentrifugal sedimentation method.[90] For the purified product they found values ranging from 12,000 to 25,000, this indicating a lack of homogeneity. From measurements on milk itself, they concluded that "purified" lactalbumin does not exist as such in milk, but, during the process of isolation, is built up from protein units, the most of which have molecular weights of less than 1000.

A number of methods have been developed for the production of soluble lactalbumin and lactalbumin preparations.[34, 124, 125] Such products usually contain considerable quantities of lactose and milk salts.

Amino acid components of lactalbumin. Table XLIV gives the relative quantities of amino acids isolated from lactalbumin. These are expressed as percentages of the amount of lactalbumin taken for the determination. Consequently, it must be remembered that, due to the water taken up in hydrolysis, complete recovery would give products totaling considerably over 100 per cent of the weight of the lactalbumin. Nitrogen distribution similarly expressed is shown in Table XLVII.

Isolation of lactoglobulin. Sebelien[96] and independently Emmerling[38] were first in the isolation of lactoglobulin from milk. It was obtained by saturating the milk with NaCl to precipitate casein, filtering, heating the filtrate to 35°, filtering off the small amount of precipitate formed, and finally saturating with $MgSO_4$. Osborne and Wakeman[88] obtained 0.52 gram of moisture-free and ash-free lactoglobulin from a liter of milk.

General physical and chemical properties. Lactoglobulin is insoluble in distilled water, but is soluble in dilute solutions of strong acids or bases or of inorganic salts. According to Osborne,[78] the hydrogen ions

Table XLIV.—Amino acid components of lactalbumin.

Monamino acids	Abderhalden and Pribram [3]	Jones and Johns [58]
	per cent	per cent
Glycine	...	0.37
Alanine	2.5	2.41
Valine	0.9	3.30
Leucine	19.4	14.03
Proline	4.0	3.76
Phenylalanine	2.4	1.25
Aspartic acid	1.0	9.30
Glutamic acid	10.1	12.89
Hydroxyglutamic acid	...	10.00
Serine	...	1.76
Tyrosine	0.85	1.95
Total monamino acids	41.15	61.02

Diamino acids and ammonia	Osborne, Van Slyke, Leavenworth and Vinograd [82]
	per cent
Cystine	1.73
Arginine	3.47
Histidine	2.61
Lysine	9.87
Tryptophane	present
Ammonia	1.31
Total diamino acids and ammonia	18.99

of the water are the cause of the globulin becoming insoluble, and this would account for the occurrence of coagulation more quickly in the presence of CO_2 or small amounts of other acids.

Lactoglobulin is a substance of a decidedly colloidal character in which the acid function predominates slightly over the basic, so that it neutralizes bases more readily than acids. According to Bechhold,[11] lactoglobulin is a pentavalent acid. Hardy [50] regards the solutions of globulins in neutral salt solutions as molecular combinations. Lactoglobulin in solution is completely coagulated at 72° and is precipitated by saturation with $MgSO_4$ and by half saturation with $(NH_4)_2SO_4$.

Osborne and Wakeman [88] consider the 0.24 per cent of phosphorus of lactoglobulin as belonging to a phospholipid, since extraction with absolute ethanol and treatment of the alcoholic residue with ether leaves a finely divided white residue having the characteristic appearance of the diamino-phosphatide obtained under similar conditions from previously coagulated milk protein. It is possible that lactoglobulin is a lecithalbumin or a mixture of proteins, one or more of which belongs to this group. Crowther and Raistrick [22] resolved by dialysis the lactoglobulin obtained from colostrum into euglobulin which is insoluble in distilled water, and pseudoglobulin which remains in solution. Hammarsten [48] doubts the individuality of these two proteins as derived from serum. Moreover,

Taylor[110] has shown that the insoluble globulin is readily converted by hydrolysis into the soluble form.

Denaturation of proteins. In the preceding discussion, the term coagulation, as applied to proteins, has been used to cover the whole process of change of a protein from a condition of solution or suspension to one of precipitation. Ordinarily, two distinguishable steps are involved, and at this point it is desirable to list the facts regarding the *irreversible chemical change* known as denaturation which precedes the *reversible physical process* which, strictly speaking, is the actual precipitation. Denaturation may be caused by heat, by light, by strong acids or alkalies, by salts of heavy metals, by violent agitation, by adsorption at a surface, by pressure, by ethanol or acetone, and possibly by lecithin. The presence of water is necessary in order for the change to take place. The

Table XLV.—Differences in properties of milk proteins.

	Casein	Lactalbumin	Lactoglobulin
Clay filters	Completely retained	At first adsorbed, then passes almost completely	Almost completely retained
Acid	Precipitated	Not precipitated	Not precipitated
Rennet	Precipitated	Not precipitated	Not precipitated
Saturated NaCl	Insoluble	Soluble	Soluble
$(NH_4)_2SO_4$	Insoluble, half-saturated	Insoluble, saturated	Insoluble, half-saturated
Saturated $MgSO_4$	Insoluble	Soluble, neutral, insoluble acid	Insoluble
Precipitin test		Each distinct[46]	
Complement fixation test.		Each distinct[10]	
Anaphylactic test		Each distinct[13, 55, 126]	

Table XLVI.—Ultimate composition of milk proteins.[117]

	Casein	Lactalbumin	Lactoglobulin
	per cent	per cent	per cent
Carbon	53.50	52.51	51.88
Hydrogen	7.13	7.10	6.96
Nitrogen	15.80	15.43	15.44
Sulfur	0.72	1.92	0.86
Phosphorus	0.71	trace	0.24
Oxygen (by difference)	22.14	23.04	24.64

Table XLVII.—Nitrogen distribution of milk proteins.[22, 83]

	Casein	Lactalbumin	Lactoglobulin
	per cent	per cent	per cent
Ammonia N	10.25	7.93	7.57
Melanin N	1.26	1.82	2.16
Cystine N	1.30	2.18	1.90
Arginine N	9.31	7.56	10.79
Histidine N	6.55	4.44	3.96
Lysine N	9.46	12.54	8.58
Amino N of filtrate	55.44	59.84	62.97
Non-amino N of filtrate	6.87	2.65	1.13

term denaturation probably covers a number of time reactions of proteins which have the common feature of causing loss of solubility in water and in dilute salt solutions at their respective isoelectric points. Denaturation will be discussed further in Chapter VIII.

Tables XLV, XLVI and XLVII recapitulate the principal differences in properties and composition of the principal proteins of milk.

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Chapter III

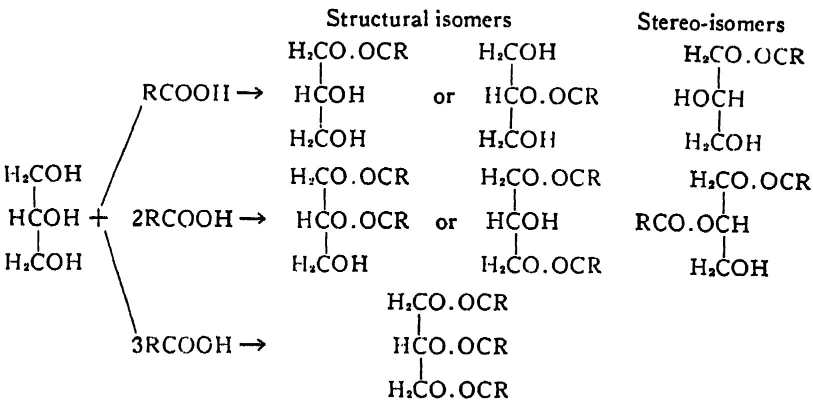
Milk Fat

Composition

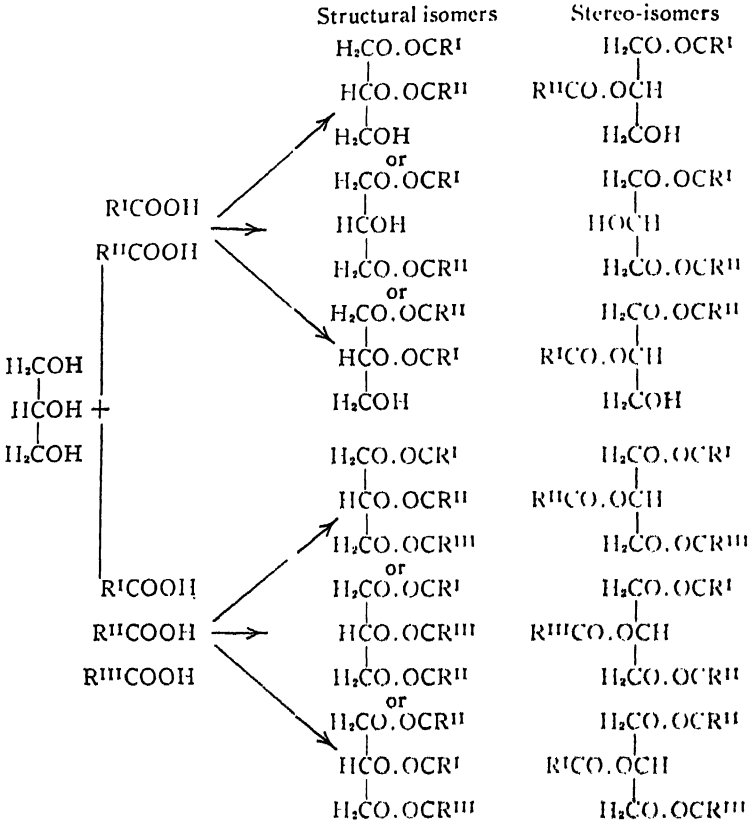
Oils and fats are essentially mixtures of the glycerides of certain aliphatic acids. Fats and oils of animal origin contain also small amounts of cholesterol, while those of plant origin are characterized by the presence of phytosterol—an isomer of cholesterol. In addition to glycerides and cholesterol, milk fat contains a natural coloring pigment—carotene (see Chapter IV), small amounts of free fatty acids and perhaps, in some instances, a trace of lecithin.

Glycerides. The glycerides are composed of the trihydroxy alcohol, glycerine, combined with various fatty acids. When one, two, or three molecules of the same fatty acid combine with one molecule of glycerine, simple mono-, di-, and tri-glycerides are formed, respectively. If two or three different acids unite with glycerine, the products formed are the mixed di- or tri-glycerides. Substitution of molecules of the same acids upon different carbon atoms of the glycerine molecule produces structurally different compounds, some of which contain an asymmetric carbon atom. A number of structural and stereo-isomeric forms of the di- and tri-glycerides are therefore possible.

SIMPLE GLYCERIDES



MIXED GLYCERIDES



Since some of the mixed triglycerides contain an asymmetric carbon atom they should be optically active. However, the only naturally occurring optically active glycerides known are those containing optically active acids.[7] Abderhalden and Eichwald[1] have synthesized optically active mono-, di-, and tri-glycerides and find that the triglycerides possess rotatory powers of very low magnitude. Some twelve or more acids are found in the glycerides of milk fat. (See p. 80.) The large number of possible combinations is apparent.

In addition to stearic, oleic and palmitic acids the glycerides contain relatively large amounts of the lower acids of the fatty-acid series, namely, butyric, caproic, capric, caprylic, lauric and myristic. They are also decomposed more easily than those of the higher acids and together with oleic acid are therefore more directly concerned in producing certain changes involved in the production of rancidity.

The low acidity, or acid value, of fresh milk fat is, according to Lewkowitsch,[61c] an indication that mono- and di-glycerides are practically absent. Many authors assert, however, that the normally low acetyl value

is a true indication of the extent to which these compounds are present. Browne[18] maintains also that because of the presence of hydroxy acids in butter-fat glycerides lactones are components of the system.

It now seems quite certain, however, that most natural fats and oils are composed chiefly of mixed tri-glycerides and that simple tri-glycerides, e.g., triolein, tristearin, etc., are practically absent.

The isolation of individual glycerides from milk fat in a pure unchanged state is extremely difficult and numerous attempts have met with little success. Employing fractional crystallization methods, Amberger[2, 3] succeeded in isolating small amounts of tristearin and triolein, and also palmito-distearin and stearo-dipalmitin. The presence of butyro-diolein, butyro-palmito-olein and oleo-dipalmitin was also suspected. Tributyrin and tricaproin were absent. Blyth and Robertson[8] claim to have isolated from milk fat a crystalline glyceride to which they ascribe the formula

$$C_3H_5\begin{array}{l} \diagup OC_4H_7O_2 \\ -OC_{16}H_{31}O_2 \\ \diagdown OC_{18}H_{34}O_2 \end{array}$$

Hydrolysis and saponification. A fat may be resolved into its components—glycerol and fatty acids—through hydrolysis with acids or alkalis, with steam under pressure, or with enzymes.

$$C_3H_5\begin{array}{l} \diagup OR^{I} \\ -OR^{II} \\ \diagdown OR^{III} \end{array} + 3H_2O \rightarrow \begin{array}{l} R^{I}OH \\ R^{II}OH \\ R^{III}OH \end{array} + C_3H_5(OH)_3$$

Water alone at higher temperatures will effect the reaction. Acids and alkalis act as catalysts. Commercially the term "hydrolysis" is synonymous with saponification since the two processes are carried out simultaneously and the fatty acids appear as the salts (soaps) of the alkali used.

$$C_3H_5\begin{array}{l} \diagup OR^{I} \\ -OR^{II} \\ \diagdown OR^{III} \end{array} + H_2O + 3KOH \rightarrow C_3H_5\begin{array}{l} \diagup OH \\ -OH \\ \diagdown OH \end{array} + \begin{array}{l} R^{I}OK \\ R^{II}OK \\ R^{III}OK \end{array} + H_2O$$

The main objections to the reactions, when carried out as indicated above, are that, in the presence of large amounts of water, they do not proceed to completion and the rate of hydrolysis is not great enough to make the reaction of practical use in analytical work. Alcoholic potash, therefore, is usually used as the hydrolyzing agent.

This procedure also allows the reaction to proceed more nearly to completion and at a greater rate because of increased solubility of the fat

in the alcohol solution. To obtain the free acids the salts are then hydrolyzed.

In analytical procedure caustic alkalis must be used if reliable results are desired. Caustic potash is slightly preferable to caustic soda because of the greater solubility of the potassium salts—soaps.

Lead oxide, baryta, lime, etc., when used as hydrolyzing agents do not complete the reaction. Hydrolysis by means of enzymes has been employed to some extent. The enzyme most commonly used for this purpose has been the lipase from the castor bean. The rate of reaction is the greatest in neutral solution and hence the liberated lower fatty acids, having appreciable degrees of dissociation, exert inhibiting effects. For rapid and more nearly complete hydrolysis by enzymes it is necessary to use the fat in the form of an emulsion.

Enzymes have a certain degree of specificity in the nature of their action and this specificity differs widely for substances of different constitution. Hence it must not be assumed that a lipase which will hydrolyze a certain glyceride, fat or oil will hydrolyze other glycerides, fats and oils with equal ease.

Cholesterol. Cholesterol occurs in small quantities in all milk fats. The results obtained by Klostermann and Opitz [52] who determined the free cholesterol before and after saponification by the digitonin method * indicate that little, if any, of the total amount is present in the form of cholesteryl esters. The values they obtained were as follows: Cholesterol in 100 grams of fat, before saponification, 75 mgs.; cholesterol in 100 grams of fat, after saponification, 71 mgs. Higher values were reported by Boemer [9] and Kirsten [50] who used extraction methods to isolate the unsaponifiable fraction. Boemer found 0.3116 to 0.4066 per cent cholesterol and Kirsten found 0.36 to 0.43 per cent. The latter author reports also that the breed and age of the animals and the type of their feeds has little influence upon the cholesterol content of the milk fat, but that with advances in the lactation period the amounts tend to increase slightly.

When milk is centrifuged, about 0.1568 per cent of the slime, of approximately 27 per cent solids content, is composed of cholesterol.[82]

Cholesterol is a monohydric alcohol containing at least one unsaturated bond. According to Windaus [91, 92] its constitution is best represented by the following structural formula:

$$\begin{array}{c} (CH_3)_2{=}CHCH_2CH_2C_{17}H_{26}CH{=}CH_2 \\ \diagup \quad \diagdown \\ CH_2 \qquad CH_2 \\ \diagdown \quad \diagup \\ CH(OH) \end{array}$$

It is insoluble in water, sparingly soluble in cold alcohol and petroleum ether, readily soluble in hot alcohol and most of the other organic solvents. It is levo-rotatory; in chloroform $[\alpha]_D = -34.3°$, and in ether $-31.1°$. It melts at approximately 146° and when heated to 300 to 320°

* Cholesteryl esters are not precipitated by digitonin.

it undergoes decomposition. At ordinary pressures it can be volatilized by careful heating and can readily be distilled unchanged in vacuum. When exposed to air and light it undergoes changes in solubility and melting point and it is not certain that oxidation is not involved in causing these variations. It forms a number of derivatives with bromine, fatty acids, ozone, digitonin, etc., and gives a number of characteristic color reactions when treated with various reagents. Of these color reactions the Liebermann "cholestol" reaction as modified by Burchard,[14] and the Salkowski reaction, are the best known. For detailed discussion of derivatives and characteristic tests the reader is referred to Lewkowitsch.[61b]

Liebermann-Burchard reaction—A little cholesterol is dissolved in 2 cc. of chloroform, 10 drops of acetic anhydride and 1 drop of H_2SO_4 are added. A violet-pink coloration which changes to blue is produced. Salkowski reaction—Concentrated H_2SO_4 is poured carefully down the side to the bottom of a test tube containing a solution of cholesterol. At the junction of the liquids a red layer is formed in the solution, which fades to a pink. The layer formed in the H_2SO_4 is of a dark red color.

Similarities in composition and reactions of cholesterol and phytosterol confused the chemistry of these compounds for some time. It is now fairly well established that they are isomers, differing, however, in crystalline form, melting point, degree of optical rotation, and melting points of their esters. The differences in physical properties between these isomers together with the observation that cholesterol is always a constituent of animal fats and phytosterol a constituent of vegetable fats and oils has led to the use of the phytosteryl acetate test as an index of the adulteration of butter fats with vegetable oils. Jaeger's [48] table of the melting points of mixtures of cholesteryl and phytosteryl acetates is reproduced here:

Table XLVIII.—Melting points of mixtures of cholesteryl and phytosteryl acetates.

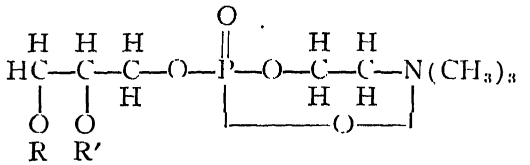
Cholesteryl acetate of m.p. 112.8° C.	Phytosteryl acetate of m.p. 129.2° C.	M.P. of mixture
per cent	per cent	degrees C.
90	10	117
80	20	120.5
73.3	26.7	122.5
60	40	125
42.4	57.6	129
20	80	129.1
10	90	129.2

The melting point of the first crop of crystals usually gives definite information as to the presence or absence of phytosterol. If phytosterol is absent the melting point of the second crop, or product recrystallized from absolute alcohol, will agree closely with that of the first crystallization. If phytosterol is present the melting point will be higher since phytosteryl acetate is less soluble in alcohol than is cholesteryl acetate.[5]

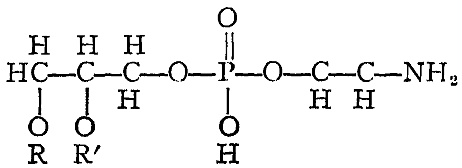
If the melting point of the fifth to seventh recrystallization lies below 115° to 116° the absence of phytosterol is a certainty.[61c]

The feeding of materials containing vegetable oils (oil cake) does not introduce phytosterol into the fat of the milk, according to the results obtained by numerous investigators.

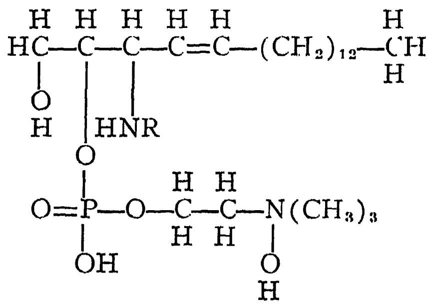
Phospholipids. The phospholipids are naturally occurring mixed glycerides of which the best known representatives have the following general chemical structures:



The Lecithins



The Cephalins



The Sphingomyelins [61]

(R and R′ represent fatty acid radicals.)

Of these compounds the lecithins and cephalins are more abundant and may occur in two structural forms, depending upon whether the phosphoric acid radical is attached to the *α* or *α′*, or to the *β* carbon atom of the glycerol radical.

The *α* glycerophosphoric acid obtained by hydrolysis of the first type would contain an asymmetric carbon atom, the *β* glycerophosphoric acid secured from the other type would not. Merz [63] has obtained both forms of glycerophosphoric acid from brain lecithin.

Both saturated and unsaturated acids have been obtained from all natural lecithins and cephalins so far investigated. Levene [59, 60] was of the

opinion that each molecule of lecithin or cephalin contained one saturated and one unsaturated fatty acid radical. However, several recent investigations have shown the existence of materials wherein both acids are saturated, and other materials wherein both acids are unsaturated.

Certain phospholipids, notably those of the tubercle bacillus and certain other bacteria, investigated by Anderson and co-workers,[4] have structures different from the formulas given above. With these exceptions, the chief differences to be found in the phospholipids from different sources lie in the nature of the fatty acid components.

The lecithins are soluble in most fat solvents, but are insoluble in methyl acetate, and in acetone, which can be used to precipitate them from solution. The cephalins show solubilities similar to those of the lecithins, but are less soluble in alcohol. The sphingomyelins are insoluble in ether and acetone, slightly soluble in cold alcohol, but easily soluble in hot alcohol. All of these phospholipids form emulsions with water from which they can be precipitated with acetone. The precipitation is facilitated by the presence of salts.

The separation of the phospholipids from each other and from neutral fat can be made by use of these solubility relationships. The separation is incomplete, however, due to intersolubilities. In the presence of large quantities of fat, the phospholipids show considerable solubility in acetone. Likewise lecithin tends to hold cephalin in solution in alcohol, and lecithin and cephalin tend to hold sphingomyelin in solution in ether.

The lecithins and cephalins are hygroscopic and readily oxidize when exposed to air. The sphingomyelins have less affinity for water and oxidize less readily.

Although Burow [15] and others had developed methods for their quantitative determination, later work by Schlossmann [83] and Njegovan [67] questioned the existence of phospholipids in milk. The work of Osborne and Wakeman [69, 70] established the fact that mixed phospholipids are present in milk in small quantities. They identified a monoamino and presumably a diamino phospholipid. Their data indicated the presence of oleic and stearic acids in the monamino fraction.

Sasaki and Hiratsuka [81] concluded that the lecithin of milk contains myristic and lauric acids, and the cephalin of milk, palmitic and lauric acids.

Koch and Woods [54] pointed out that lecithin and cephalin are associated with each other in all animal tissue and maintained that cephalin is a constituent of milk and occurs in amounts varying from 0.027 to 0.045 per cent, the maximum value of which is approximately equivalent to the lecithin concentration reported by them.

In a recent extensive investigation of the constitution of the phospholipids isolated from dry buttermilk, Kurtz [56] has found the following ratio of lecithin to cephalin to sphingomyelin, 8.4 : 4.5 : 1. Cerebrosides were found in a quantity about equal to that of the sphingomyelin, and in the same fraction of the phospholipids.

Analysis of the lecithin-cephalin fraction showed the following com-

position of the fatty acids: Myristic acid, 5.23 per cent; stearic acid, 16.06 per cent; arachidic acid, 1.85 per cent; oleic acid, 70.58 per cent; dicostetrenoic acid, (?), 6.28 per cent.

Although the identity of the highly unsaturated acid could not be proved, a variety of analytical data indicated it to be a C_{22} acid with four double bonds.

Figures obtained by various authors for the lecithin content of milk vary greatly in value. Since the results in practically all cases are based upon the phosphorus content of extracted materials, these variations must be due to incompleteness of extraction of phospholipid material in some cases and to extraction of inorganic phosphorus in others. In the Table XLIX only those publications which are most complete in scope and in which the results are most representative of those generally found in the literature have been cited. A complete bibliography may be obtained by consulting articles by Mohr and Moos,[65] and Holm, Wright and Deysher.[46]

Table XLIX.—Phospholipids in some milk products according to various authors.

Author	Whole milk	Skimmed milk	Cream	Butter	Butter-milk	Separator slime
	per cent	per cent	per cent	per cent	per cent	per cent
Dornic & Daire [24].	0.0595	0.0332	.....	.....	0.0651 0.0944	.....
Bordas & Raczkowski [10]	0.0909 0.0252	0.018	0.334 (50%)*	.....	.....	.....
Hess & Helman [36].	0.1819 0.1377	0.1072 0.1156	0.2668(32%)* 0.1859(20%)*	.....	.....	.....
Chapman [18]	0.0345 0.0709	0.0082 0.029	0.1824 0.2155	.....	0.1036 0.148	.....
Cusick [19]	.....	.....	.....	0.0433 0.0723	.....	.....
Rewald [75]	0.05	0.0096	.....	0.95	.....	.....
Laxa [57]	0.06	.....	.....	.....	.....	0.2562 0.3017
Grimmer & Schwarz [82]	.....	.....	.....	.....	.....	0.419
Mohr & Moos [65]...	0.0038 0.2889	0.0015 0.150	0.05 0.334	0.014 1.60	0.0332 0.8768	.....
Mohr, Brockman & Müller [64]	0.037	0.0155	0.1685	0.2060	0.1142	.....
Holm, Wright & Deysher [46]	0.158	0.129	0.269	0.247	0.318	.....

* Fat content of cream.

Recent work in the author's laboratory seems to indicate that the values for the phospholipid content of milk of the order of those obtained by Mohr, Brockman and Müller [64] represent the phospholipid content of milk most accurately.

The destruction of lecithin in milk by heating was studied by Bordas and Raczkowski,[10] who found that at 95° to 110° for 30 minutes the loss of lecithin was approximately 30 per cent. Mohr, Brockman and Müller found, however, that pasteurization had little effect upon the lecithin content of milk. That pasteurization of cream has but a slight effect upon

the lecithin content of the butter was noted by Cusick,[19] who noted also that pasteurized ripened cream produced a butter of lower lecithin content than did the raw ripened cream.

The phospholipids have been thought to be of extreme importance as intermediary substances in the process of fat utilization in the animal body. Their universal presence in the tissues and the apparent increase in lipid phosphorus in the blood during fat absorption appeared to be an indication of their importance and probable function, respectively.

Jordan, Hart and Patten [49] found that a cow's diet low in phosphorus content caused a lower milk fat production than when the phosphorus supply in the diet was adequate.

Meigs, Blatherwick and Cary [62] studied the phospholipid content of jugular and arterial blood and concluded from their studies that milk fat originates mainly if not entirely in the phospholipids of the blood. Later work (see p. 568) by others has shown however that this view is incorrect.

Fatty Acids

The presence of appreciable amounts of the volatile and somewhat soluble acids of the acetic series is especially characteristic of milk fat and confers upon it certain properties which serve to distinguish it from other fats or oils. These characteristics vary somewhat according to the proportion of the different acid constituents, which in turn is dependent upon the type of feed of the animals. Some of the properties of these fatty acids are shown in Table L.

As stated previously, the amounts of fatty acid constituents are subject to considerable variations, depending upon numerous factors, chief among which is the type of feed of the animals producing the fat.

The most complete analyses of milk fat to date are those of Holland et al,[38] and Hilditch and Sleightholme,[37] which are given herewith.

The presence of arachidic and linoleic acids is the principal feature wherein the analyses by Hilditch and Sleightholme differ from those of Holland et al., Frog and Schmidt-Neilsen,[26] and Browne.[12] Browne's results differ also from those of the former in the report of the presence of dihydroxy stearic acid to the extent of 1.00 per cent.

The results of Hilditch and Sleightholme indicate a seasonal variation in the composition of the milk fat. Later work by Dean and Hilditch,[22] in which the winter diet of the animals was normal, substantiated these observations. They found that, within a short time of the cows' return from winter feeding to pasture, the amount of unsaturated fatty acids increased about 4 per cent (molal) while the saturated acids decreased correspondingly. This decrease was chiefly of butyric and stearic acids. Dean and Hilditch found also that the composition of the milk fat produced varies with the age of the cow. With increasing age of the animal the proportion of unsaturated fatty acids gradually increases, mainly at the expense of the palmitic acid.

Burr and Burr [16] observed that butter did not readily cure the nutri-

Table L.—Physical constants of fatty acids.

Acid	Formula	Melting point	Boiling point 760 mm	Boiling point Reduced pressures	Solubility in 100 cc. Water	Solubility in 100 cc. Alcohol	Solubility in 100 cc. Ether	Volatility (steam)
		° C.	° C.	° C.	grams	grams	grams	
Acetic series:	$C_nH_{2n}O_2$							
Butyric	C_3H_7COOH	—7	163	......	Sol.	Sol.	Sol.	Volatile
Caproic	$C_5H_{11}COOH$	—8	202	......	$0.882^{15°}$	Sol.	Sol.	Volatile
Caprylic	$C_7H_{15}COOH$	16.5	236	......	$\left\{ \begin{array}{c} 0.079^{15°} \\ 0.25^{100°} \end{array} \right\}$	Sol.	Sol.	Volatile
Capric	$C_9H_{19}COOH$	31.3	268	114 (15 mm)	$0.10^{100°}$	Sol.	Sol.	Volatile
Lauric	$C_{11}H_{23}COOH$	43.6	>300	176 (15 mm)	V. sl. sol. at 100°	$134^{21°}$	Sol.	Appreciably vol.
Myristic	$C_{13}H_{27}COOH$	54	...	196.5 (15 mm)	Insol.	$44.9^{21°}$	Sol.	V. s. vol.
Palmitic	$C_{15}H_{31}COOH$	63	...	215 (15 mm)	Insol.	$9.90^{20°}$ V. sol. (hot alc.)	Sol.	Non-vol.
Stearic	$C_{17}H_{35}COOH$	69.3	...	232 (15 mm)	Insol.	$2.0^{20°}$ V. sol. (hot alc.)	Sol.	Non-vol.
Arachidic	$C_{19}H_{39}COOH$	77	...	......	Insol.	$0.45^{20°}$ Sol. (hot alc.)	Sol.	Non-vol.
Oleic series:	$C_nH_{2n-2}O_2$							
Oleic	$CH(CH_2)_7CH_3$ ‖ $CH(CH_2)_7COOH$	16	...	223 (10 mm)	Insol.	Sol.	Sol.	Sl. vol. with superheated steam at 250° C.
Linoleic series:	$C_nH_{2n-4}O_2$							
Linoleic	$CH{=}CH(CH_2)_4CH_3$ | CH_2 | $CH{=}CH(CH_2)_7COOH$	<—18	...	228 (14 mm)	Insol.	Sol.	Sol.	Sl. vol.

Table LI.—Fatty acids in 21 samples of butterfat from Massachusetts cows fed normal rations. (From Holland et al.)

Acid	Range	Av.
	per cent	per cent
Butyric	2.241- 4.230	2.932
Caproic	1.290- 2.400	1.898
Caprylic	.527- 1.041	.786
Capric	1.187- 2.008	1.570
Lauric	4.533- 7.687	5.849
Myristic	15.554-22.618	19.784
Palmitic	5.782-22.863	15.167
Stearic	7.803-20.370	14.907
Oleic	25.273-40.313	31.895

Table LII.—Summary of fatty acid analyses (molar percentages) of butterfats. (From Hilditch and Sleightholme.)

Acid	I (Autumn-fed, 1928)	II (Cocoanut cake, 1929)	III (Soya cake, 1929)	IV (Early summer pasture, 1929)	V (Pasture-fed December, 1928)
	per cent	per cent	per cent	per cent	per cent
Butyric	8.4	9.0	9.6	8.9	9.2
Caproic	3.5	3.9	3.0	2.7	3.4
Caprylic	2.7	1.7	2.8	2.0	2.2
Capric	2.9	4.3	5.1	3.0	4.2
Lauric	4.1	8.3	7.5	4.7	4.7
Myristic	7.2	17.2	10.7	10.9	11.5
Palmitic	27.1	24.1	23.7	24.3	25.0
Stearic	6.4	3.9	6.7	5.4	9.5
Arachidic	0.7	...	0.9	...	0.5
Oleic	33.9	25.7	27.0	34.6	26.1
Linoleic	3.1	1.9	3.0	3.5	3.7

tional disease caused by a fat-free diet and which is cured by the acids more highly unsaturated than oleic. Eckstein [25] has investigated the unsaturated acids of milk fat and has isolated bromides characteristic of the tetra-, hexa-, and octo- compounds, and reported the presence of 0.19 to 0.52 per cent of linoleic acid and 0.11 to 0.15 per cent of linolenic acid, the higher amounts being found in milk fats produced when linseed meal was a constituent of the animal feed. Due to the difficulty of making a quantitative separation of the bromides, the values reported are probably low.

Smedley,[85] from an apparent maximum in the iodine number of the C_{10} fraction of the methyl ester fraction inferred the presence of decenoic ($C_{10}H_{18}O_2$) acid. Later Grün and Wirth [33] isolated this acid in amounts equivalent to 0.01 per cent of the milk fat used and showed that its double bond existed between the 9th and 10th carbon atoms.

Bosworth and Brown [11] have fractionated large quantities of the methyl esters of the fatty acids of milk fat and confirmed the presence of decenoic ($C_{10}H_{18}O_2$) acid and reported also the presence of tetradecenoic ($C_{14}H_{26}O_2$) and the saturated acid, lignoceric ($C_{24}H_{48}O_2$). They also obtained evidence for the probable occurrence of C_{20}, C_{22}, or C_{24} acids with

two double bonds and highly unsaturated acids of the arachidonic type. They failed, however, to identify linoleic acid.

It is interesting to note that all the acids found in the glycerides of milk fat and those of practically all animal fats contain an even number of carbon atoms, although fatty acids containing odd numbers of carbon atoms are utilized readily by the animal body. The assumption that the fatty acids are built up as they are broken down—two carbon atoms at a time—seems to be supported by these facts.

Other constituents. Small amounts of free fatty acids are always present in fresh milk fat. Greater amounts are found in the fat isolated from milk or cream that has been subjected to bacterial action or in fat that has been stored for some time.

The characteristic flavor and aroma of milk fats have been generally attributed to these acids and to the glycerides containing the lower fatty acids. Recent work has shown, however, that the characteristic butter aroma may have its origin in a minor constituent. Niel, Kluyver and Derx [66] isolated acetylmethylcarbinol from butter and noted a direct correlation between the quantity present and the intensity of the butter aroma. Since freshly prepared acetylmethylcarbinol is odorless they concluded that the aroma was due to biacetyl which was present to the extent of 0.0002 to 0.0004 per cent and which was probably formed from acetylmethylcarbinol during the ripening process of the cream. Schmalfuss and Barthmeyer [84] found biacetyl in butter to the extent of 0.0001 to 0.0006 gram of its derivative nickel-biacetyl-dioximin per kilogram of butter and Davies [21] reports biacetyl present in butter to the extent of 0.05 to 0.50 parts per million.

The fatty acids of the milk fat of other species. Though the milks of various species may differ considerably in their percentage compositions of various constituents, those of the acid constituents of their fats are quite similar as shown in Table LIII.

Table LIII.—Molar distribution of the component acids of cow, goat, sheep, camel, and buffalo milk fats.

	Cow * [37]	Goat [23a]	Sheep [23a]	Camel [23b]	Buffalo [6]
Butyric	8.9	7.6	8.4	5.9	11.0
Caproic	2.7	4.5	5.4	1.9	2.8
Caprylic	2.0	6.2	5.8	1.1	1.5
Capric	3.0	11.1	10.1	2.1	2.3
Lauric	4.7	5.1	6.0	5.7	3.3
Myristic	10.9	11.2	11.8	7.9	10.4
Palmitic	24.3	21.5	20.4	28.3	28.7
Stearic	5.4	7.3	5.4	9.7	9.3
Arachidic	...	.1	1.3	...	.7
Oleic	34.6	24.2	22.3	34.1	27.8
Linoleic	3.5	1.2	3.2	3.3	2.2

* Early summer pasture fed.

Separation and estimation. Several lines of procedure have been followed in the separation of the various acids, as follows:

(a) Crystallization of the acids from various solvents.
(b) Separation of various salts through solubility measures.
(c) Fractionation of the acids by vacuum distillation.
(d) Fractionation of methyl and ethyl esters of the acids.
(e) Separation of the acids by steam distillation.

Each method is complicated by the fact that in the presence of one another the chemical and physical characteristics of the acids (solubility, volatility, etc.), and of their derivatives are altered considerably.

Fractionation of the lower fatty acids by crystallization is extremely difficult, especially in the presence of oleic acid. The first of these methods is feasible, therefore, only when an estimate of the quantities of the higher acids of the saturated series is desired.

Of the various salt solubility methods that have been tried the lead-salt-ether method has met with the greatest success. The lead salt of oleic acid is soluble in warm ether and therefore affords a method for separating this unsaturated acid from palmitic and stearic acids, whose lead salts are practically insoluble in ether. The method is open to the objection, however, that the solubilities of the lead salts of the solid saturated acids are greatly increased in the presence in solution of the lead salt of oleic acid. For a detailed discussion of the various attempts at separation through the salts of barium, potassium, lithium, silver, etc., the reader is referred to Lewkowitsch [61a] and Paal and Amberger.[71]

Fractionation of the acids by vacuum distillation [55] has failed as an analytical procedure. Fractionation with steam gives only the approximate

Table LIV.—Melting and boiling points of esters.

Acid	Methyl ester		Ethyl ester	
	M. P.	B. P.	M. P.	B. P.
	° C.	° C.	° C.	° C.
Butyric	....	102	—93.3	120
Caproic	....	150 52-3 (15 mm.)	....	167
Caprylic	—40 (s.p.)	192-4 83 (15 mm.)	—48 (s.p.)	207-8
Capric	—18 (s.p.)	223 114 (15 mm.)	....	242
Lauric	5	141 (15 mm.)	—10 (s.p.)	269 79 (*vacuo*)
Myristic	18	167 (15 mm.)	11 (s.p.)	295
Palmitic	28-9	196 (15 mm.)	24	185 (10 mm.)
Stearic	38-9	214-5 (15 mm.)	36.7	139 (*vacuo*)
Arachidic	54.5	....	54	....
Oleic	....	212-3 (15 mm.)	....	....
Linoleic	....	207-8 (11 mm.)	....	....

s.p. = solidifying point.

values for the lower volatile saturated acids, and is complicated by the fact that oleic acid is also volatile with steam. Of the distillation methods those for the separation of the methyl or the ethyl esters of the acids have

furnished the most satisfactory results. The principal difficulty encountered is the same one encountered in all distillation methods, namely, lack of a clear cut separation of the various fractions, though with the esters this is less pronounced. The oleic acid esters appear in all of the fractions, but may be corrected for through calculations based upon the iodine values of each fraction. Combinations of crystallization and distillation methods have furnished the most complete analysis.

Methods of Examination of Milk Fats

Of the physical methods for the examination of fats, the determination of the refractive index, the melting and solidifying points, and the microscopic appearance have proved the most useful.

The microscopic appearance of a fat has been employed chiefly in the detection of beef fat, lard, and cocoanut oil. In the investigation of the unsaponifiable matter this method has been of invaluable assistance, especially in the detection of vegetable fats and oils, through detection of phytosterol, or its derivatives.

The chemical methods generally used in the study of fats furnish, however, the best information as to the true nature of the product.

Detailed discussion of each method is beyond the scope of the allotted space, hence the brief discussion of the different methods given here. (See Reference 5.)

Refractive index. The determination of the refractive index, though rapid, does not afford a reliable method for detecting adulterations; yet it may be used as a rapid test to decide whether or not adulteration may be suspected.

Melting point, solidifying point. The determinations of melting and solidifying points have presented difficulties due largely to lack of care and uniformity in methods. From the standpoint of physical laws the melting point and solidifying point of a glyceride should be identical, since they are reciprocal phenomena which occur at the same temperature. When melted substances solidify the latent heat of fusion is liberated and a slight rise in temperature takes place. Large quantities of the acids show this temperature rise very well, but fats show a very slight change. This retarded velocity of change in temperature of a fat with changes of the temperature to which it is subjected may explain the great variations in melting point and solidifying point figures obtained by various workers. In determining the melting point and solidifying point of any fat the rate of change in bath temperature should, therefore, be exceedingly slow.

"Double melting point." In the case of many glycerides the phenomenon of "double melting point" has been observed. Some glycerides when heated in a capillary tube melt at a certain temperature and, on further heating, become turbid and finally melt again at a higher temperature. The phenomenon has been explained by some workers as being due to the existence of isomeric forms. These forms are known to exist in

isolated β-lauro-α-α'-distearin, β-myristo-α-α'-dilaurin, β-myristo-α-α'-distearin and other glycerides. Of the isomeric forms that exist one is usually the stable and one the labile form, and during heating the tendency is toward a total conversion into the stable isomer. Glycerides that have previously been heated, therefore, usually exhibit one melting point—that of the stable modification. Glycerides of different melting and solidifying points greatly affect each other in this respect. Free fatty acids, when present, always modify these values.

In view of the facts stated it is evident that the melting point of a milk fat is very indefinite. The fat is not only a mixture of glycerides of different acids, but each glyceride may exhibit some of the peculiar characteristics enumerated.

Turbidity test. The turbidity test has proved of some value in the detection of adulterants. However, due to the fact that the properties of milk fat vary greatly with the type of feed, it is questionable whether a method based upon solubilities can furnish an accurate clue to deliberate adulteration.

Acid value. The acid value is equivalent to the number of milligrams KOH necessary to saturate the free fatty acids in one gram of fat. This test indicates the amount of fatty acids not combined with glycerine.

Reichert-Meissl value. The Reichert-Meissl value is equivalent to the number of cubic centimeters of decinormal alkali necessary to neutralize the volatile acids from 5 grams of fat. The Reichert-Meissl value indicates the extent to which the lower or volatile fatty acids are present. Milk fat contains a greater amount of these fatty acids than does any of the fats from which it might be desirable to distinguish it and hence furnishes a most valuable index in its examination. The value varies considerably, however, from season to season, with various countries, and with other conditions already discussed elsewhere.

Saponification value (Koettstorfer). The saponification value (Koettstorfer) is equivalent to the number of milligrams KOH required to saponify one gram of fat. A butterfat that yields a high R.M. value also gives a high saponification value though the latter may drop considerably in the last stages of lactation. The two tests should be collated. A high saponification value and a low R.M. value are grounds for suspicion of adulteration.

Soluble, and insoluble acids (Hehner number). The percentages of soluble and insoluble acids present, calculated as butyric, is known as the Hehner number. This test indicates proportion of higher and lower fatty acids in fat. The value of the test lies in the fact that butterfat is characterized by a greater amount of soluble acids than other fats.

Polenske value. The number of cubic centimeters of $N/10$ $Ba(OH)_2$ required to neutralize the volatile fatty acids insoluble in water is known as the Polenske value. Most volatile fatty acids in milk fat are soluble in water and hence this test serves to distinguish between this fat and a fat of high caprylic acid content (cocoanut oil).

Jensen-Kirschner value. The Jensen-Kirschner value is equivalent to the number of cubic centimeters of $N/10$ alkali required to neutralize a distilled Ag_2SO_4 filtrate from 100 cc. of Reichert-Meissl distillate. This value is a measure of the butyric acid in fats. The presence of butyric acid is especially characteristic of milk fat.

Acetyl value. The acetyl value is equivalent to the number of milligrams of KOH required for the saponification of the acetyl assimilated by one gram of fat on acetylation (Holland). This value is a measure of the free hydroxyl (OH) groups in a fat.

Cadmium value. The cadmium value is a measure of the amount of lower fatty acids in a fat, especially butyric, caproic, and caprylic.[72]

Iodine value (Hanus method). The per cent of iodine absorbed by a fat is known as its iodine value (Hanus method) and is a measure of the proportion of unsaturated acids in a fat,—in milk fat, oleic acid, and perhaps some linoleic acid.

Unsaponifiable matter. The substances in a fat which are not soluble in water and do not combine with KOH to form soaps are referred to as unsaponifiable matter. In milk fat this is composed mainly of cholesterol.

Halphen test. The Halphen test is a color reaction obtained with the addition of amyl alcohol, carbon bisulfide and one per cent sulfuric acid. It is largely used to detect the presence of cottonseed oil. Since the substance in cottonseed oil which gives the test is destroyed in various ways, a negative result should not be accepted as proof that cottonseed oil is absent.

Baudouin test. A color reaction obtained with the addition to a fat of cane sugar and hydrochloric acid is known as the Baudouin test and is used to detect sesame oil. Turmeric and some organic dyes, when used as coloring matter, also give the test.

Cottonseed and sesame oil cake increase the milk yield but have the tendency to impart to the milk fat produced some of the properties of their fats. Thus, sesame oil cakes when fed produce a soft butter, and in many cases impart to the milk fat the property of giving a Baudouin oil test. Cottonseed oil cake when fed imparts to milk fat the property of giving the Halphen reaction.

It should be remembered here, however, that the phytosteryl acetate test furnishes a method to detect adulteration, since phytosterol does not pass into the milk fat when vegetable oils are fed. A positive phytosteryl acetate test must, therefore, mean that vegetable oils have been added directly.

Kreis test. When an ether solution of phloroglucin, and concentrated hydrochloric acid, are shaken with fat that has become oxidized, a reddish pink color is produced in the aqueous layer. This is known as the Kreis test. The intensity of the color is dependent upon the degree of autoxidation of the unsaturated acids, especially oleic.

Other tests. Other tests sometimes mentioned but of little practical value are the Elaidin test, Bromide test, Maumene thermal test, etc.

Normal range of values. In view of the fact that the acid distribution in the milk fat glycerides is subject to extreme variations no definite numerical values for the physical and chemical tests described can be given. The values given in Table LV must, therefore, be considered only as indicating approximate ranges of values.

Table LV.—Range of characteristic milk fat values.

Sp. Gr. 37.8°C / 37.8°C	M.P. °C	Solidifying point °C	Refractive index 40°C	Refraction Butyro scale	Mean mol wt.	Free acid value	Iodine number	Jensen Kirschner number	Acetyl number	Reichert Meissl number	Insol acids Hehner number	Polenske number	Saponification value
Approx. .9100-	28-	19-	1.4527-	40.5-	258-	Of fresh milk fat	26-	20-	1.9-	23-	86.5-	1.50-	220-
.9200	36	24.5	1.4566	46.	266	0.56	38	26	8.6	33	90	3.00	241

It should be remembered, however, that in spite of the wide range over which the results of the tests may vary when different milk fats are used, several of the tests give values that are especially characteristic of milk fats only.

Deterioration of Milk Fat

General discussion. Undesirable changes occurring in dairy products containing milk fat produce conditions which may be classed as rancid, tallowy or fishy. Much confusion has arisen concerning the use of the first two terms. However, it is generally agreed, especially among workers in the field of dairy products, that the term "rancid" should be applied to hydrolytic changes only, while the term "tallowy" should be used where oxidation changes are the cause of off odors and flavors, excluding changes that produce "fishiness." In other fields of work, where the types of acids present in the fats concerned are different from those found in milk fat, the differences in off odors and flavors produced in deterioration are less sharply marked and hence the term "rancid" is used almost exclusively. As a rule the various changes involved occur simultaneously in most fats and oils and no sharp line of differentiation can be drawn.

Milk fat, on account of its relatively high percentage of the lower fatty acids, especially butyric, readily produces a strong odor characteristic of these acids, upon slight hydrolysis. Most other fats contain relatively small amounts of the lower acids and large amounts of higher acids, stearic, palmitic, etc., which are odorless. Hydrolysis of these fats yields, therefore, comparatively little of the off odors.

Oxidation of the unsaturated acids with subsequent splitting and formation of aldehydes, acids and ketones is the cause of tallowy odors and flavors. Oxidation of oleic acid is mainly responsible for intense tallowy odors.[39] Oxidation of linoleic acid produces less intense off odors, while oxidation of linolenic acid produces a very slight amount of off

odors. Oxidation of oleic acid in the presence of either of the latter seems to lessen the intensity of the off flavors.[42]

It is clear, therefore, that milk fat presents a combination of fatty acids which makes possible intense rancidity, or tallowiness, independently. Slight hydrolysis produces rancidity due to the high content of lower fatty acids and slight oxidation produces intense tallowy odors characteristic of the autoxidation products of oleic acid. In deteriorated milk fat we have, therefore, the result of each of the two reactions magnified considerably over the results produced in other fats by analogous reactions.

Rancidity. The production of free fatty acids in some dairy products is due primarily to hydrolytic action by enzymes or the action of free acids in the product, in the presence of water. The accelerating effect of the free acids is undoubtedly analogous to their action in the acid hydrolysis of sugars and dependent upon the H-ion concentration.[80] The lower acids (butyric, caproic, etc.) produced by this hydrolysis are responsible for the rancid odors and flavors. Other changes due to oxidation may occur simultaneously.

The rate of enzyme action is largely a function of temperature, hence the control of deterioration by enzymes is at least partially successful. Through pasteurization of milk or cream it is possible to reduce this action in the products manufactured to a point where it no longer becomes a great industrial menace. The ordinary pasteurization temperature should render the lipases, or fat-splitting enzymes, inert. There is a question, however, as to whether or not the holding process (63° for 30 minutes) entirely destroys enzyme activity.

Though the major part of the responsibility for acceleration of hydrolytic changes in some instances may be ascribed to enzyme action, it should be remembered that the initial acid content, of the product dealt with, also influences the rate of hydrolysis.

In order to produce butters possessing flavor and aroma the practice of ripening the cream to be used with chosen cultures, thereby producing acidity of the degree desired and enhancing aroma, has been general. While producing a product of the acidity and flavor preferred by some consumers the compounds formed are objectionable from the standpoint of increased susceptibility of the product to oxidation changes.

The fundamental work of Rogers and Gray [76] formed the basis for the solution of the problem of deterioration of butter in storage. These authors concluded from the results of their work that sweet cream butter was superior in keeping quality to butters made from ripened creams.

To reduce the acid content of a highly acid cream the practice of neutralization has been generally resorted to. The great danger in this practice lies in lack of control. Overneutralization of a cream produces a butter of inferior keeping quality, especially from the standpoint of the ease with which oxidation changes are initiated. The extent to which hydrolysis is promoted during neutralization is dependent upon the chemical nature of the neutralizer used. Because of these hydrolytic changes

that may be initiated by the high degree of acidity and increased enzyme activity, and those produced subsequently during neutralization, a butter produced from a highly acid cream must be viewed as inferior to one made from cultured cream, which in turn is inferior to sweet cream butter, for storage.

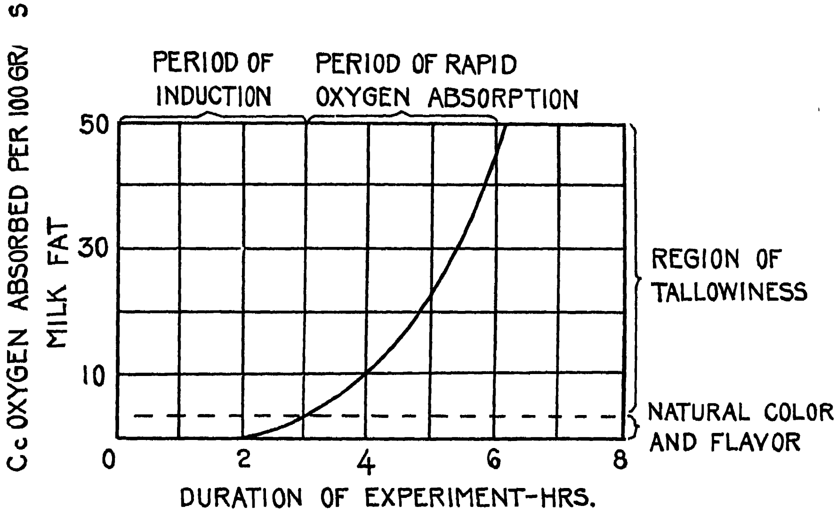


Fig. 1.—The nature of oxygen absorption at 95° C. and the changes that occur in the different stages of the absorption.

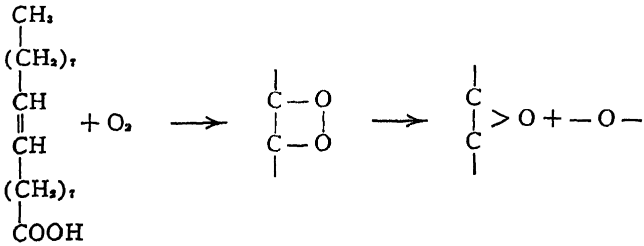
Tallowiness. It has long been suspected, and has been stated by some authors, that oleic acid is the main constituent of fats concerned in the production of tallowy odors and flavors, although the relative value of the factors causing the changes has been the subject of considerable controversy.

This acquired defect is due mainly to an oxidation of the oleic acid radical. Holm and Greenbank [39, 41, 42] have shown that when small amounts of oxygen are taken up by milk fat or oleic acid a tallowy product is produced.

Milk fat does not absorb oxygen as soon as it comes in contact with it, or with the atmosphere, but passes through a period during which there is very little or no absorption, called the induction period. At lower temperatures this period is considerably longer than indicated in Figure 1.

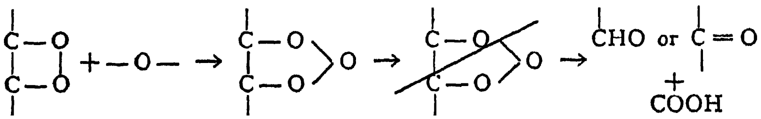
As time progresses in this period the susceptibility of the fat to oxidation increases. While not identical with conditions of storage it is analogous to such conditions and a measure of the length of this period furnishes a valuable index of the keeping quality of a fat.[39, 40]

The reactions which occur during this period are not well understood. There occurs a slight oxygen absorption with, presumably, a formation of compounds of increased oxidizing intensity. Several reactions undoubtedly occur simultaneously, the general natures of which may be as follows:



Oleic acid Peroxide Oxide and active oxygen

According to Tschirch and Barben[80] the active oxygen unites with the peroxide to form an ozonide which is very unstable and breaks up to form one molecule of an aldehyde, or the corresponding ketone, and one molecule of an acid, as indicated.



The above formulae represent schematically some of the reactions that probably occur. There is a possibility that hydrogen atoms, necessary to complete some of the reactions occurring in active oxidation, come from other groups in the chain thereby producing additional unsaturated bonds which are immediately oxidized.

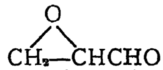
In view of the number of saturated aldehydes and acids isolated by Scala[82] from oxidized fats, it seems that the oxidation is a progressive formation and oxidation of unsaturated bonds. As soon as splitting of the molecules occurs substances of a catalyzing nature are formed and oxygen absorption becomes rapid, proceeding at a logarithmic rate. (See p. 89.) Absorption of relatively small amounts of oxygen produces tallowy odors and flavors and, therefore, prevention of deterioration through oxidation is a problem of retarding or preventing the initial changes,—or keeping the state of the fat within the induction period.

Though the exact nature of the reactions involved in the various phases is not known, it is known that peroxides are formed progressively, the rate of their formation depending upon the temperature, acidity of the product, its previous history of treatment, etc. During the early stages of the autoxidation and prior to the stage of active oxidation the amount of these peroxides is an indication of the relative ease with which a fat will produce tallowy flavors and odors in storage.[30, 58]

The Kreis color reaction is produced by a milk fat as soon as active oxidation begins and its intensity bears a quantitative relationship to the amount of oxygen absorbed.[41, 42] The probable chemical nature of the compounds responsible for the test was demonstrated by Powick.[78] This author found that acrolein plus hydrogen peroxide produced a compound

which gave a positive Kreis reaction. Epihydrin aldehyde also produced a positive reaction. These facts support the idea of a progressive oxygen

$CH_2=CHCHO$
acrolein


epihydrin aldehyde

addition to and subsequent splitting of the oleic acid radical as indicated previously. (See p. 90.)

The development of peroxides and perhaps other compounds of great oxidizing intensity in a fat introduces the possibility of subsequent reactions involving the saturated acids present and those formed. Dakin[20] has shown that β oxidations of these compounds may occur in the presence of hydrogen peroxide, according to the theory of Knoop.[53]

$$RCH_2CH_2COOH \rightarrow RCH(OH)CH_2COOH \rightarrow R\overset{\displaystyle O}{\overset{\|}{C}}{-}CH_2COOH \rightarrow$$
$$R\overset{\displaystyle O}{\overset{\|}{C}}{-}CH_3 + CO_2$$

Raper,[74] and Cahen and Hurtley[17] have shown that oxidations of the γ and δ carbon atoms are also possible. The possibility of the presence of a large number of compounds in an oxidized fat is evident.

Among the many factors that have been mentioned as accelerators in the production of tallowiness in milk fat are heat, light, acidity, moisture, enzymes and metals. Metals are active catalysts for many reactions in organic chemistry. The use of finely divided nickel in the hydrogenation of oils, platinum in the sulfuric acid process, alumina and thoria in dehydration reactions, etc., are familiar examples. The use of various pigments (metal oxides) as driers is another well known example of catalysis by metallic compounds. Copper, because of the ease with which it passes from one state to another, has long been known as an excellent catalyst for the oxidation of organic compounds. Other oxides possessing marked catalytic oxidizing activity are those of iron, platinum, silver, nickel, cobalt, vanadium, cerium, chromium, uranium and lead.

Contamination of milk products with small amounts of various metals, especially copper or iron, rapidly induces oxidation with a consequent production of tallowiness. Small amounts of copper or iron incorporated in butter produce rapid deterioration.[47, 79]

Copper salts added to milk to the extent of 10-15 parts per million parts of milk solids accelerate the production of tallowiness in its powder.[88]

Because of the ease with which copper is attacked, the use of copper equipment in the handling of milk and its products is ever a potential source of danger.

Iron has been found to be less active as a catalyst for oxidations. Tin has but a slight tendency to pass into solution when used in equipment

for the handling of milk and is much less active than copper or iron as an oxidation catalyst, and is therefore used extensively as a lining for copper equipment.

Increased temperatures and ultraviolet light as well as certain bands of light of the visible spectrum are markedly catalytic in their action.[31]

Much work has been done by Rogers and his associates upon the effect of acidity upon the keeping quality of butter. The general conclusions arrived at were that acidity has a decided effect upon keeping quality and that sweet cream butter always possesses a better keeping quality than does butter made from ripened cream. They also conclude from their studies that enzyme action plays a minor rôle in the changes in the flavors of butter. In summary Rogers [78] concludes—"that the principal changes in the flavor of butter are probably due to spontaneous chemical changes in which oxidation plays an important part" and that "the action of micro-organisms is indirect and the major change is brought about by spontaneous chemical reaction."

Increases in the acidity of a fat increase its susceptibility to oxidation.[42]

Conflicting views have been held for some time as to the exact rôle of water as a catalyst for the oxidation of fats. It is of interest to note that Winckel [90] was not able to obtain rancidity tests upon butter treated similarly to other fats. Holm and Greenbank [40] noted that extremely dry whole milk powders showed "off" odors and flavors long before similar powders of higher moisture content. High moisture contents in powders tend to produce fishy flavors. Figure 2 illustrates the relations of moisture content and vapor pressure of a powder to changes that occur.

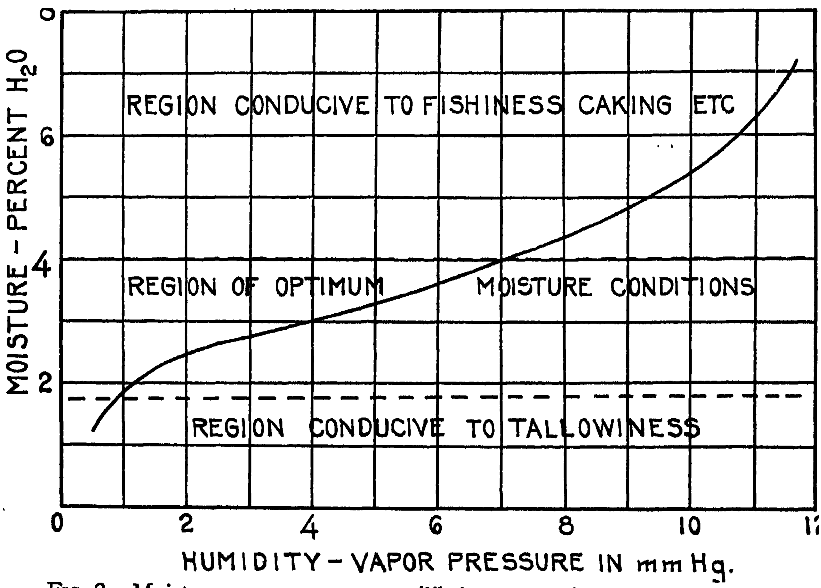


FIG. 2.—Moisture-vapor pressure equilibrium curve for a dry whole milk.

Subsequent work [28] by the latter authors also proved that the presence of moisture had a retarding action upon the oxidation process and probably prevents the formation of aldehydes and ketones which are the stable products and produce the tallowy odors and flavors in oxidation in a dry medium. In a humid atmosphere the end products are presumably acids of no tallowy odor. Since acids are catalysts [28] for the oxidation reactions it should be expected that after a preliminary stage the rate of oxidation would be rapid. This was shown to be the case in milk powders of relatively high H_2O content. The moisture content of milk powder has been shown to be a critical factor in determining the rate of oxidation in this product. In view of the fact that different powders have different avidities for moisture, studies and comparisons should be made at moisture contents where vapor pressures are the same, especially if results are to be judged by the olfactory sense.

Any neutralization method which will in any way promote hydrolysis must be avoided if the best results are to be obtained from the standpoint of reducing susceptibility of the product to oxidation.

Other factors than those already mentioned enter into the keeping quality of whole milk powders. Holm, Greenbank and Deysher [43, 44] have determined the changes of susceptibility of whole milk powders manufactured under various conditions and have concluded that:

(1) Increased fat content of the powder increases the susceptibility of the product to oxidation. Above 24 per cent fat content the increase in susceptibility is rapid with additional fat increases.

(2) Condensation of the milk improves the keeping quality of its powder slightly, while condensation and homogenization of the milk markedly improve the keeping quality of its powder.

(3) Clarifying of the milk before powdering produces a powder of markedly superior keeping quality. This is due to removal of slime, since when the slime is returned to the milk it produces a powder no better than that produced from the unclarified milk. Clarification is, therefore, the plausible explanation for the excellent keeping quality of powdered cream.

(4) Temperature of pasteurization greater than the ordinary temperature in use improves the keeping quality of the powder.

The effect of the various temperatures of storage upon the rate of oxidation in various dairy products has received little consideration from a quantitative standpoint. Holm, Wright and Greenbank [45] have followed the changes in susceptibility of the fat in milk powders to oxidation during storage at various temperatures above 0° and have noted marked improvement in the keeping quality at temperatures near 0° over that at 10°. Storage at 20° caused relatively rapid deterioration when compared with the rate of deterioration at the lower temperatures.

Results of the work of Gray and McKay,[27] and of Rogers, Thompson and Keithley [77] indicate that for prolonged storage of butter a temperature of −18° (0° F.) should be maintained.

These conclusions agree with experimental results obtained by the present authors upon the effect of different temperatures upon the

peroxide formation in pure fats. At temperatures of -10° or lower there is little or no increase in the peroxide value over long periods of storage. The rate increases progressively with increases in the temperature.

The use of so-called inert gases has received serious consideration in connection with the problem of storage of dairy products. It should be mentioned here that carbon dioxide, which has received greatest consideration, will hydrolyze soaps in the presence of water and probably acts similarly upon glycerides. The results obtained by Holm, Wright and Greenbank [45] upon milk powders stored in CO_2 seem to indicate that CO_2 can not be considered an inert gas toward dairy products containing milk fat.

Storage in nitrogen or in vacuum reduces slightly the tendency to deterioration but should not be considered an absolute safeguard to prevent oxidation changes.

Fishiness. This defect usually occurs in butters but may also develop in milk powders of too high moisture content. The fact that the moisture content is high wherever this condition is encountered suggests hydrolytic action as a factor in its cause.

The works of Supplee,[88] Cusick,[10] and Sommer [86] have established the fact quite conclusively that this defect is caused by hydrolysis of lecithin producing trimethylamine,

$$N \begin{array}{l} \diagup CH_3 \\ - CH_3 \\ \diagdown CH_3 \end{array}$$

which is the compound responsible for the fishy flavor.

$$\text{Lecithin} + H_2O \rightarrow \text{glycerophosphoric acid} + \text{choline} + \text{acid.}$$
$$\text{Choline} + H_2O \rightarrow \text{trimethylamine} + \text{other compounds.}$$

Former views were that bacterial decomposition is directly responsible for the condition.[34, 35, 68] In view of later work it seems probable that enzyme action is only indirectly concerned.

Pasteurization of a cream lessens the tendency of its butter to become fishy. In view of the work of Supplee [87] and of Dornic and Daire [24] which showed that pasteurization lowers the lecithin content, Sommer [86] ascribes this to hydrolysis of lecithin and to its losses through the buttermilk. Rogers and Gray [76] found that the addition of acids to cream tends to produce fishiness in its butter. Since slight acidities favor the hydrolysis of lecithin it is evident that acidity is a factor. Rogers states that he has never encountered fishiness in unsalted butters and other investigators state that it is a defect mainly of salted butters. Sommer [86] holds that since a salt solution is a better solvent for lecithin than is water, it is probable that during working of butter, and especially in cases of overworking, lecithin is concentrated in the salt solution wherein water and acidity would have their maximum effect. The cause for this tend-

ency in renovated butter is perhaps analogous. The rôle that oxidation plays in this reaction must be indirect and slight, though it may accompany this change. Perceptible oxidation in the product would render it unfit for consumption before the action upon lecithin could become involved.

Metals accelerate the production of fishiness through catalysis of the hydrolysis reaction.

In view of what is known concerning the reactions producing rancidity, tallowiness, and fishiness, and the factors involved, it may be said that, to insure good keeping quality, prime importance should be placed upon the "freshness" of the fat in the product.

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Chapter IV

Pigments of Milk *

General Discussion

The white or "milky" appearance of cow's milk is caused by the scattering of reflected light by the fat globules, the colloidal calcium caseinate, and the colloidal calcium phosphate in the milk. Dispersions of each of these ingredients are milky when prepared separately in concentrations similar to that occurring in milk, and, in the case of the fat, with the particles the size of the globules in milk.

Despite the milky appearance of the fat dispersion, the fat globules of cow's milk contain more or less yellow pigment dissolved in the fat. This gives the milkiness more or less of a yellow tinge, depending on the concentration of pigment in the fat. This color becomes more pronounced when the fat globules are concentrated, either by gravity or centrifugal force, as in cream. It becomes still more apparent when the cream is churned into butter and is most evident when one views the melted, filtered butter fat.

Skim milk also contains a yellow pigment, or pigments, masked by the colloidal dispersions of casein and calcium phosphate. This pigmentation becomes evident when the colloidal dispersions referred to are destroyed by coagulation with rennin or acid, the latter precipitating the casein and dissolving the calcium phosphate. The whey or serum obtained by filtering off the coagulated material always has a more or less yellow color with a distinct greenish fluorescence. This is caused by the pigment or pigments dissolved in the aqueous phase of milk.

It is apparent, therefore, that milk contains two classes of pigments, fat soluble and water soluble. Among the former, carotenoids only are found, and of these carotene [51] predominates. The same pigment characterizes the adipose tissue and skin secretion of dairy cattle, especially the Jersey and Guernsey breeds. It is not at present clear whether more than one water soluble pigment occurs in cow's milk. Blyth,[5] and Bleyer and Kallmann,[4] although evidently dealing with different chemical substances, called the pigment lactochrome. A recently [12, 34] suggested name is lactoflavin, which is regarded as one member of a larger group of specific flavins occurring in nature to be collectively called lyochromes. Whether only one [36] or several lactoflavins or lactoflavin compounds [18] exist naturally in cow's milk is somewhat uncertain. One or another of the sub-

* Revised to March 1, 1934.

stances recently isolated in crystalline form undoubtedly represents the pure form of the lactochromes studied by the earlier workers.

Carotene and lactoflavin are entirely different substances, bearing no relation to each other either with respect to their chemistry or their origin in milk. Therefore, it seems best to discuss them separately.

Fat-Soluble Pigments

Carotene. Carotene belongs to a class of pigments called carotenoids which are widely distributed in plants and are found in many animals. For a comprehensive review of their distribution see the monograph by Palmer.[46] The more recent developments in this field are excellently summarized by Zechmeister.[78] A chemical isomer of carotene, called lycopene, causes the red color of tomatoes, watermelons, the nonedible fruits of certain plants and a few flowers, but has not been shown to occur in animals, definitely not in milk. Carotene apparently always has several other members of the carotenoid group associated with it in plants. Formerly all of these were collectively known as xanthophylls but are now designated by specific names, such as xanthophyll, lutein, zeaxanthin, etc., although the nomenclature employed by various investigators * is not uniform and therefore somewhat confusing. Green plant tissues, especially leaves, usually contain only about one-half as much carotene as the xanthophyll group of carotenoids. Some of the latter accompany carotene in milk fat, but always in very minor proportions.

The carotenoids are synthesized by plants, but not by higher animals. The occurrence of these pigments in milk fat is due therefore to their direct transfer from the food. Experiments [51] published in 1914 first demonstrated that the concentration of carotene in the milk fat and blood serum of dairy cows varies directly with the amount of carotene in the food. A summary [46] of the first group of experiments showing these relationships is given in Table LVI. More recent data,[2, 19] giving quantitative estimations of carotene and "xanthophyll" [probably xanthophyll (lutein) plus zeaxanthin] of butter fat in relation to season and ration, are summarized in Table LVII.

On examining the data in Table LVI it should not cause surprise that the timothy hay employed was a carotene-poor food. Its green color was negligible, as is frequently the case. The explanation of the insignificant effect of yellow corn lies in the fact that the predominating pigments of yellow maize are xanthophyll (lutein) and zeaxanthin, not carotene. The former pigments occur in pigmented butter fat in relatively much smaller quantities than carotene, even under the most favorable conditions, as the data in Table LVII show.

Of the mammals whose milk is commonly used for human food, cows alone secrete milk which is characterized by a pronounced pigmentation of the fat. Milk fat from the goat, ewe, camel, and water buffalo is

* The specific pigment called xanthophyll by Karrer [28] is the same as the lutein of Kuhn,[37] this being the principal oxygen-containing carotenoid in green plants, in many seeds and the one occurring in greatest amount in the yolks of hens' eggs.

Table LVI.—The relation between carotene-rich and carotene-poor rations and the color of milk fat and blood serum.

Breed of cow	Ration	Butter Fat		Blood Serum	
		Yellow	Red	Yellow	Red
	Carotene-poor rations				
Ayrshire	Cottonseed meal and cottonseed hulls....	1.3*	0.4	3.3**	0.5
"	Cottonseed hulls, timothy hay and white corn	1.2	0.4	2.6	1.1
"	Cottonseed meal, cottonseed hulls, timothy hay and yellow corn.................	2.0	0.5	4.9	1.2
Holstein	Cottonseed hulls, corn stover and cottonseed meal	8.5	1.4	6.0	0.7
"	Cottonseed hulls, corn stover and cottonseed meal	3.0	0.7	7.0	0.8
Ayrshire	Cottonseed hulls, corn stover and cottonseed meal	2.5	0.6	11.0	0.9
Jersey	Cottonseed hulls, corn stover and cottonseed meal	11.0	1.7	10.0	0.9
"	Cottonseed hulls, corn stover and cottonseed meal	5.2	1.2	13.0	1.1
"	Cottonseed hulls, corn stover and cottonseed meal	4.7	1.5	7.5	0.7
	Carotene-rich rations				
Ayrshire	Cottonseed meal, cottonseed hulls, timothy hay, yellow corn and carrots....	24.0	1.3	54.0	1.8
"	Mixed grain, green alfalfa hay and fresh pasture grass	16.0	1.1	40.0	1.0
Holstein	Mixed grain, green alfalfa hay and fresh pasture grass	54.0	1.8	48.0	1.1
"	Mixed grain, green alfalfa hay and fresh pasture grass	22.0	1.2	41.0	1.0
Jersey	Mixed grain, green alfalfa hay and fresh pasture grass	64.0	2.0	45.0	1.1
"	Mixed grain, green alfalfa hay and fresh pasture grass	54.0	1.7	57.0	1.8
"	Mixed grain, green alfalfa hay and fresh pasture grass	47.0	1.6	45.0	1.0

* The color of the butter fat was determined by matching a 1-inch layer of rendered melted fat with the color glasses of the Lovibond tintometer.

** The color of the blood serum was determined by matching the extract from 10 cc. of serum in 12.5 cc. volume and 1-inch layer with the color glasses of the Lovibond tintometer.

practically colorless, if not entirely devoid of pigment. The fat of human milk, however, is at times [54] distinctly tinted by carotenoids. The fundamental reason for this difference is not known. It has been shown,[45] however, that pigmented milk fat or adipose tissue occurs only in those species whose blood serum is colored by carotenoids. Further than this there is no definite physiological information available to explain why cow's milk has this special characteristic.

An equally inexplicable fact is the failure of xanthophyll and its related carotenoids to be absorbed by dairy cattle in quantities proportionate to their occurrence in the food. The experiment with yellow corn cited in Table LVI shows that xanthophyll (lutein) and zeaxanthin are not transformed into carotene by cattle. The same fact is undoubtedly true for the other carotenoids whose chemical composition is like that of

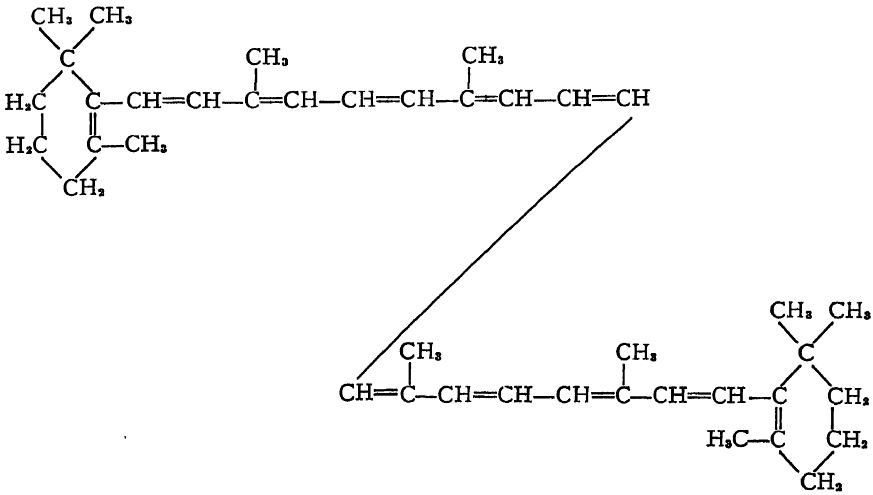
Table LVII.—Quantitative estimations of carotene and xanthophyll in butter fat in relation to character of feed.

Character of Ration	Carotene per 100 grams dry butter		100 grams dry butter		No. Samples	Investigator
	Mean	Range	Mean	Range		
	mg.	mg.	mg.	mg.		
Winter ration of hay, roots and concentrates	0.275	0.0606 to 0.613	0.0223	0.0069 to 0.072	11	Gillam and others [19]
Grass silage, hay, roots and concentrates	0.208	0.0465 to 0.521	0.0191	0.00685 to 0.0747	9	Gillam and others [19]
Hay, roots, artificially dried nitrogen-treated grass and concentrates	0.462	0.119 to 0.703	0.0295	0.00963 to 0.0629	10	Gillam and others [19]
Hay, roots, artificially dried non-nitrogen-treated grass and concentrates	0.347	0.0882 to 0.491	0.0181	0.003 to 0.0301	8	Gillam and others [19]
Late winter and early spring rations	0.22	0.20-0.24	....	....	4	Baumann and Steenbock [2]
Summer pasture	0.785	0.71-0.86	....	....	2	Baumann and Steenbock [2]
Late pasture and fall rations	0.440	0.40-0.58	....	....	4	Baumann and Steenbock [2]
Winter rations	0.240	0.20-0.30	....	....	4	Baumann and Steenbock [2]

xanthophyll (lutein) and zeaxanthin. The greater ease of destruction of these carotenoids in the digestive tract of cattle and their greater solubility in the bile of this species, observed [58] a number of years ago, still remain the only clues to the enigma. That the absorption of carotene from the digestive tract of cows is relatively small on abundant carotene intake has long been known [58] and recently [42] confirmed. Xanthophyll (lutein) and zeaxanthin, also, are not completely destroyed.[25]

Constitution and properties. Carotene is an unsaturated hydrocarbon with the empirical formula $C_{40}H_{56}$. Its constitution has been completely established since the first edition of this Monograph, at which time practically nothing was known regarding the chemical structure of the pigment. Carotene, in common with the other carotenoids, may now be defined [78] as a fat-soluble, water-insoluble, nitrogen-free aliphatic polyene pigment, whose color is caused by a long system of conjugated double bonds. Furthermore, the conjugated unsaturated carbon chain is methylated at such intervals as to indicate that it is composed of a series of four reduced (dehydrogenated) isoprene groups and is thus chemically

related to the terpenes and phytol. At each end of this chain a β-ionone ring is attached. The structural formula[26] of the optically inactive β-carotene is thus



β-carotene

β-carotene appears to be the most widely distributed carotene in nature. It is probably the only form of carotene normally occurring in butter fat. An optically active carotene, α-carotene, occurs in carrots, red palm oil and in certain leaves, usually in minor quantities*; its maximum $[\alpha]^{18°}_{607.5}$ in benzene $= +485°$. The asymmetric carbon atom of α-carotene is the carbon atom of one of the rings to which the aliphatic polyene chain is joined. Two other isomeric forms of carotene occur[71] in plants. These have been called γ- and δ-carotene, respectively. The former is optically inactive and differs structurally from β-carotene in having one of the ionone rings open. δ-carotene is believed to be an analogous isomer of α-carotene, retaining the optically active ring. Both γ- and δ-carotene are thus structurally related to the optically inactive lycopene in which both ionone rings are open. An artificial "isocarotene," differing markedly from the other isomers in the position of its spectroscopic absorption bands, has been prepared from β-carotene tetraiodide.[35]

Carotene, being a hydrocarbon, does not form esters. The carotenes thus exist as free chemical compounds in plant tissues and animal fats, including butter fat, in contrast to the oxygen-containing xanthophyll group of carotenoids which occur in nature both free and as esters. A naturally occurring dipalmitic acid ester of zeaxanthin has been named physaliene. Heleniene is a like ester of xanthophyll (lutein). The esters are readily hydrolyzed to liberate the pigments. Since this occurs also

* The carotene in tea leaves is said to be exclusively α-carotene.

in the animal body the free pigments only are found in such species as absorb them.[33] Several halogen derivatives of carotene have been prepared, namely, the complete substitution and addition compound with bromine $C_{40}H_{36}Br_{22}$, a pure addition compound with bromine $C_{40}H_{56}Br_{16}$, a complete iodo-chloride addition product $C_{40}H_{50}Cl_{11}I_{11}$, and also di-, tri-, and tetra-iodide addition compounds. The presence of carotene in butter fat thus contributes slightly to the iodine number.

Highly purified carotene crystals are fairly stable against oxidation, even in the air, although autoxidation eventually causes a gradual loss of color after which an increase in weight occurs until as high as 37.87 per cent oxygen may be absorbed. Impure crystals and solutions [2, 44] in many fat solvents, as well as in fats and oils, are much more sensitive to oxidation. The bleaching of butter in the tallowy decomposition of the product is caused by the oxidation of the carotene present and is, in fact, an indicator of the general chemical character of this butter decomposition. By very gentle oxidation with chromic acid, β-carotene has been changed to β-oxycarotene (orange red needles, m.p. 184°) and to β-carotenone (carmine color hexagons, m.p. 174 to 175°).[32] The former contains one opened ionone ring, the latter has both rings opened, each with the addition of two atoms of oxygen.

The facility with which carotene undergoes oxidation, especially in solution, has long been regarded as a possible clue to one of its functions in nature. The question whether carotene acts as an antioxidant or a prooxidant is of interest in connection with the tallowy (oxidative) decomposition of butters containing different amounts of this pigment. Although the question has not been studied for solutions of carotene in butter fat, experiments with lard,[44] linoleic acid [15] and cottonseed oil [20] to which carotene has been added * show that the pigment is an active prooxidant even when present in concentrations from 15 to 150 times the maximum concentration of carotene so far observed in butter fat. On the other hand, the carotene in butter fat reduces added ferric chloride to the green ferrous salt when suitably small amounts are dissolved [55] in warm melted fat, the test serving as a striking color reaction for the pigment. Other carotenoids also give this reaction.

The supposition has been advanced [61] that butter fat contains natural antioxidants, which are destroyed or removed by charcoal, and which normally protect the carotene from oxidation. The experimental observations upon which this supposition rests may be explained also on the basis that the charcoal treatment induces in the fat an autoxidation which is accelerated by the prooxidizing power of the carotene itself, when added to the decolorized fat, the pigment thus assisting its own destruction.

Both α- and β-carotene may be catalytically hydrogenated. Dihydrocarotene,[62] $C_{40}H_{58}$, is an oily, pale yellow product. The completely hydrogenated product, perhydrocarotene,[74] $C_{40}H_{78}$, is a white, transparent,

* The antioxidant activity of carotene towards linoleic acid, observed by Monaghan and Schmitt,[40] is explained by Franke [15] as due to the fact that the linoleic acid employed was first strongly oxidized before the carotene was added. The high concentration of carotene, i.e., nearly 0.5 per cent, may have been another factor.

crystallizable product having very few of the characteristic properties of carotene. Perhydro-α-carotene is still dextrorotatory.

Carotene is usually regarded as a yellow pigment. The color, however, depends upon whether one is referring to the crystals or solutions. The latter vary in shade and intensity of color with the concentration of pigment, the nature of the solvent, and the stereoisomeric form. During crystallization, e.g., from methanol, the crystals have a deep copper hue, and on isolation the matted crystals are violet, those of β-carotene being darker violet. When dissolved in any of its solvents, except carbon disulfide, carotene is yellow to orange to deep red as the concentration increases, the color of the β-carotene solutions being 1.3 times as intense as the α-carotene. In carbon disulfide the pigment has a red hue in all concentrations, the more dilute being pink, the shade of color deepening to rose red, blood red and reddish black, as the concentration increases.

Carotene crystals have never been obtained from butter fat because the pigment can not be obtained in concentrated form from the fat by simple extraction as can be done from plant materials. Crystals of carotene from carrots or leaves have a rhombic or prismatic form, or are indented quadratic leaflets. From alcohol the crystals usually contain ½-⅔ mol of alcohol of crystallization which is given up in vacuum over H_2SO_4 or P_2O_5. The crude crystals melt at 174° (corrected), but after repeated recrystallization both the α- and β- forms melt at 183 to 184° (uncorrected).

All carotenoids give color reactions with numerous reagents, most of which are either strong acids or chlorides of polyvalent elements. Blue is the dominant color given in these reactions although in some cases green is evident and in other cases violet. These reactions are not specific for carotenoids but are given by all substances with the polyene structure. A detailed description of each of these numerous tests would be out of place in this monograph, although a brief account of the reaction with antimony trichloride in chloroform solution is probably warranted because of its extensive use in recent years as an indicator of vitamin A activity. When 1 to 2 mg. carotene dissolved in 1 cc. dry chloroform is treated with 2 cc. of 28 per cent solution of $SbCl_3$ in chloroform, a fairly stable dark blue, violet-tinted compound is formed. This compound in chloroform shows two characteristic absorption spectrum maxima at 588 mμ and 542 mμ. The presence of oil does not interfere with the formation of the colored compound. Naturally colored butter fat, therefore, gives an indication of this test because of the presence of carotene. The color fades rather quickly when the test is applied to butter fat. There are several reasons for this, the more important being: (a) only an insignificant fraction of a milligram of carotene is present in the few drops of butter fat employed for the test, as usually carried out; (b) vitamin A, the nearly colorless half molecule of carotene, apparently always present in butter fat in higher concentration than carotene, forms a similar but much less stable compound with $SbCl_3$. The $SbCl_3$ reaction of butter fat when sufficiently

stabilized for quantitative colorimetric readings is therefore always greater than can be accounted for by the amount of carotene present.[30]

Carotene is not attacked by alkalis. The fact that carotene in alcoholic solution withstands boiling with strong KOH or NaOH without loss of properties made possible the first[51] demonstration of the carotenoid nature of the butter fat pigment, because the carotene could be separated unaltered from the fat in the unsaponifiable ether-extractable fraction of the aqueous solution of the butter fat soaps.

The various isomeric forms of carotene dissolve readily in melted fats and oils containing liquid glycerides, as well as in various fat solvents, including ether, chloroform, petroleum ether, carbon disulfide, cyclohexane, benzene, ethylene dichloride, etc. A saturated solution in ethyl ether (one of the moderately good solvents) contains 0.1 per cent. Petroleum ether is a somewhat better solvent for α-carotene than for the β-form. Ordinary aliphatic alcohols are poor solvents, and carotene is practically insoluble in pure ethanol and methanol. Carotene is not extracted by 85 to 90 per cent methanol from its petroleum ether or carbon disulfide solution, although the latter solvents will quantitatively extract carotene from methanol-petroleum ether or CS_2-petroleum ether solutions to which sufficient water is added to dilute the alcohol to 85 to 90 per cent alcohol and cause the separation of the solvents into two layers. The unesterified forms of the oxygen-containing carotenoids having the carotene structure, namely, xanthophyll (lutein) and zeaxanthin, in general show the opposite behavior between 85 to 90 per cent alcohol and petroleum ether or carbon disulfide. The diluted alcohol will extract the largest part of them from the petroleum ether or carbon disulfide provided glycerides are not present to assist their solubility in the better fat solvents. When these relative solubility methods of separating carotene from xanthophyll and its related pigments are applied to the unsaponifiable matter from normally (not artificially) colored butter fat it is possible to demonstrate [51,19] that the normal carotene of butter fat is accompanied by minor quantities of the oxygen-containing carotenoids.

The various carotenoids are characterized by distinct differences in adsorption affinity towards various adsorbents, when dissolved in petroleum ether or carbon disulfide. The adsorbent formerly employed most extensively, namely precipitated $CaCO_3$, has been replaced in recent years by fullers' earth, CaO and $Ca(OH)_2$ powder, alumina and fibrous alumina. Carotenes, in general, are not appreciably adsorbed from petroleum ether by these agents although α- and β-carotene, because of a difference in adsorption affinity, may be separated when their solution in ligroin is treated with fullers' earth [31] or especially when their solution in petroleum ether is filtered through $Ca(OH)_2$ or CaO [29] in a chromatographic analysis, i.e., filtration of a given quantity of suitable pigment solution through a column of the adsorbent so that the differentially adsorbed pigments separate into zones. β-carotene is more readily adsorbed in the foregoing procedures than is α-carotene. A few of the many other interesting adsorption differences of the carotenoids, when petroleum ether is the

solvent, are as follows: Lycopene is adsorbed by fibrous alumina, the carotenes are not; xanthophyll (lutein) is readily adsorbed by $CaCO_3$, zeaxanthin much less readily but more readily than carotene. Elution of adsorbed pigments is effected with methanol.

By employing various combinations of the relative solubility and adsorption properties of the carotenoids an almost complete separation of the several pigments is possible.[31]

Carotene solutions are decolorized by various decolorizing charcoals. Butter fat, dissolved in petroleum ether, is thus decolorized[67, 47] but the elution of the pigment from the charcoal is not possible; destruction of the carotene by oxidation undoubtedly occurs. Certain preparations of alumina and also silica gel act nearly as well as charcoal as adsorbents[23] of carotene from petroleum ether solution. Carotene exists in the blood of cows as an adsorption complex with serum albumin.[52] The pigmented protein is readily isolated. It disperses readily in water and this solution, as well as the dry substance, does not give up the carotene to fat solvents unless first treated with alcohol.

Each of the pigments at present classified as a carotenoid shows typical spectroscopic absorption bands. Three bands are visible in the blue and violet region of the spectrum.

The typical positions of these bands for each carotenoid differ somewhat with different solvents, depending on their index of refraction. Carbon disulfide, because of its high refractive index, shifts the bands into a brighter region of the spectrum where they become more easily discernible when solutions of proper concentration are employed, i.e., about 0.5 mg. per 100 grams. Formerly it was possible to distinguish the absorption spectra of carotenoids only by the approximate wave lengths of the light rays of the spectrum at which the boundaries of the absorption bands occur when like solutions of pure pigments were examined by means of a suitable spectroscope. With the development of spectrophotometers a more accurate differentiation of the carotenoids became possible, through measurement of the wave length at which the absorption bands show their maximum intensity of absorption with a suitable, constant light source. Furthermore, detection of bands (absorption maxima) in the ultra-violet became possible by use of properly sensitized photographic plates. By this more delicate method it has been possible both to differentiate[73] the various isomeric forms of carotene and also to show that carotene possesses two absorption maxima in the ultra-violet, in addition to the three maxima in the visible spectrum.

Pure β-carotene, the chief carotenoid of butter, shows five maxima in $CHCl_3$ solution at 495, 463, 436, 348 and 280 mμ, which are shown in Figure 3,[19] in terms of log I_0/I (I_0 = intensity of incident light from an underwater tungsten spark, I = intensity of emergent light) at various wave lengths. The unsaponifiable matter from butter shows an identical curve[2, 48] of intensity of absorption in the visible spectrum, the two maxima in the ultra-violet being masked by a much greater absorption intensity of other substances, the major one of which is vitamin A.[2, 43]

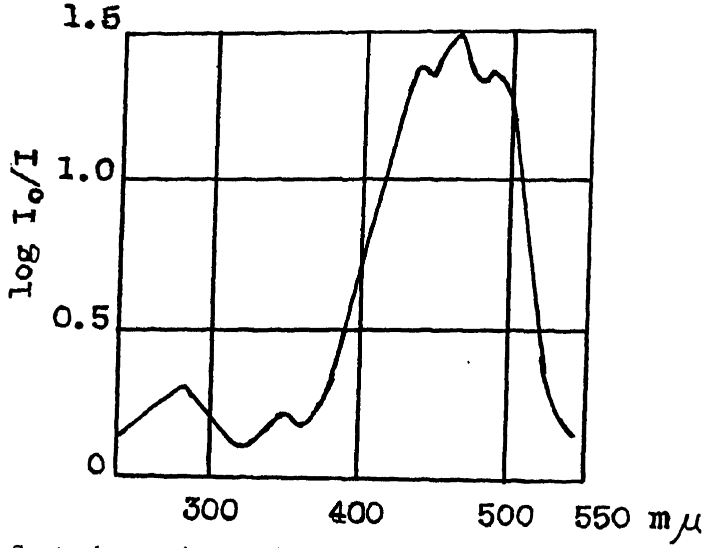


FIG. 3.—Spectrophotometric transmittancy of 0.00078 per cent solution of carotene in chloroform through one cm. layer, according to Gillam, Heilbron, Morton, Bishop and Drummond.[19]
Abscissa: Wave lengths in mμ. Ordinates: transmittancy in terms of log I_0 (intensity of incident light) ÷ I (intensity of emergent light).

Isolation of carotenoids from milk. Inasmuch as the fat of cow's milk contains all of the carotenoids present in the milk, the dry filtered fat from carefully melted (at temperatures not to exceed 45 to 50°) butter is by far the most suitable starting material for the isolation of the milk carotenoids. The procedure to be followed is based on the fact that the carotenoids will appear quantitatively in the unsaponifiable matter of the fat. The precautions to be observed are: (1) to effect complete saponification of the glycerides, (2) to prevent the formation of colored resinous material which will be extractable with the unsaponifiable matter, (3) to avoid oxidation of the carotenoids in the purification of the unsaponifiable extracts or in their evaporation and handling prior to the submission of the pigments to examination or analysis. It is not advisable to employ large quantities of fat for the isolation.

The procedure with regard to the amount of fat, character and strength of alcoholic potash used for saponification, length of saponification period, extent of dilution of soap with water, number of extractions of the soap with ether, character of drying agent employed for the ether extract, use of oxygen-free atmosphere, etc., varies considerably in different laboratories.[2, 6, 19, 38, 51]

Nearly all of the chemical and physical characteristics of the carotenoids may be demonstrated with ease when using the unsaponifiable matter from well-colored, naturally pigmented butter fat. If it is desired to ascertain whether representatives of the xanthophyll group of

carotenoids are present, a petroleum ether or carbon disulfide solution of the pigmented material may either be submitted to a chromatographic analysis or an equal volume of methanol added to these solutions and then sufficient water to cause the solvents to separate. In the latter, so-called phase separation both layers will usually be colored showing the presence of both carotene (in the petroleum ether layer) and members of the xanthophyll group (in the alcoholic layer).

The amount of carotene that has been isolated from butter fat by the above general procedure has not exceeded about 0.8 mg. per 100 grams fat, as shown in Table LVII. The maximum concentration possible under the most favorable conditions of feeding and breeding of cattle, has not been determined. It is doubtful that it will ever be found to equal that in deeply pigmented, mature carrots, which may contain [8] as much as 10 mg. per 100 grams fresh tissue.

Quantitative determination of carotene in butter fat. The first method proposed [48] for estimating carotene in butter fat was the Willstätter-Stoll colorimetric method using 0.2 per cent $K_2Cr_2O_7$ solution as standard. When applied directly to the filtered melted fat, a value of 4.85 mg. carotene per 100 grams fat was obtained for a sample from cows on fresh green pasture. Values of 4.0 and 3.6 mg. carotene per 100 grams butter fat for two samples of Guernsey butter and 1.8 mg. for a sample of Ayrshire butter fat have been obtained [70] by this method, the animals being on the same ration.

Another method of determining carotene in butter fat is based on the assumption that the color reaction with $SbCl_3$ is due wholly to the carotene present and also represents the total vitamin A activity. The carotene content of butter fat that may be deduced from results [38, 58] based on this assumption ranges from 6 mg. to 16 mg. per 100 grams. It has been shown,[42, 43] however, that butter fat contains both true vitamin A and carotene, the former in larger proportions; this method of estimating carotene content of butter fat is obviously inaccurate inasmuch as both carotene and vitamin A give the $SbCl_3$ reaction. Lundborg,[39] who found that carotene added to butter fat could be estimated colorimetrically with $K_2Cr_2O_7$ using petroleum ether solutions of the fat, showed that the carotene present naturally in the fat, estimated by this method to be 1.25 mg. per 100 grams fat, was much greater (7.9 mg.) when estimated by the blue $SbCl_3$ reaction given by the unsaponifiable matter in the fat. He apparently did not appreciate, however, that this large difference was due to the relatively high concentration of vitamin A in comparison with carotene.

The $K_2Cr_2O_7$ colorimetric method for fairly pure carotene extracts or solutions has been severely criticized by Schertz [59] who recommended the spectrophotometric method because of its much greater accuracy. Apparently, however, pure carotene in petroleum ether solution may be estimated [61] with nearly equal accuracy by the two methods, at least in certain concentrations (about 2 mg. per 100 grams). However, when butter fat is present, the estimation may be 60 per cent to 114 per cent too high, for

some as yet unexplained reason. In view of the limited distribution of facilities for spectrophotometric estimation as compared with facilities for colorimetric estimations, further studies seem warranted of direct colorimetric methods for estimating carotene in butter fat. Color standards other than $K_2Cr_2O_7$ should be studied, e.g., the mixed aqueous solution of Naphthol Yellow (2,4-dinitro-α-naphthol sulfonic acid) and Orange G (benzene-azo-β-naphthol-γ-disulfonic acid) recommended by Sprague[68] (3.4 cc. 1 per cent solution of the former plus 0.5 cc. 1 per cent solution of the latter, added to 1 liter of water), and especially the 0.0145 per cent solution of azobenzene employed by Kuhn and Brockmann.[38] The color of solutions of this dye shows a rectilinear relationship to the color of various concentrations of carotene solutions instead of the curvilinear relationship exhibited by $K_2Cr_2O_7$ solution.

For the spectrophotometric estimations of carotene in butter fat, Baumann and Steenbock[2] compare the absorbency ($\log I/I_0$) of the melted butter fat at 30°, at 460 mμ and 485 mμ against a standard solution of carotene in cottonseed oil using a Bausch and Lomb universal spectrophotometer, and calculate the concentration of carotene by direct proportion. The accuracy of the method has been verified by correctly estimating known amounts of carotene added to butter fat and oleomargarine. The inconvenience of keeping the fat melted, as is necessary when using some spectrophotometers, and the fact that cottonseed oil is difficult to free from light absorbing substance prompted the following modifications (unpublished) of the procedure in the author's laboratory. Petroleum ether solutions of the butter fat and carotene standards are employed and butter fat, completely decolorized by charcoal, is used in place of cottonseed oil for the carotene standard with a small amount of added hydroquinone to protect the carotene from oxidation.

Gillam and associates[19] calculate the carotene concentration in the epiphasic (petroleum ether) fraction of the unsaponifiable matter of a given amount of butter fat by determining the molecular extinction coefficient at 463 mμ photographically, using a sector photometer, and compare the result in direct proportionality ratio to the standard value E (molecular extinction coefficient) = 102,000. The Baumann-Steenbock procedure disregards the presence of xanthophyll and zeaxanthin, as do the colorimetric procedures, but the data so far obtained by it and by the photographic procedure show essentially the same range of values, as shown in Table LVII.

Variations in carotene content of milk and feeds. The amount of carotene in butter fat is not constant. The colostral milk of all dairy cattle, and of humans is very highly colored with pigment. In the case of cow's milk, well-colored fat will continue to be formed only if the food contains an abundance of carotene. This fact explains the seasonal variation in the natural color of butter and at the same time raises a question as to which cattle feeds yield an abundance of carotene and which do not. A classification of the common dairy cattle feeds from this point of view is as follows: Carotene-rich feeds—green pasture grass; hay, cured to

retain a rich green color, such as most Western-cured alfalfa; all soiling crops; green corn fodder; very new corn silage; carrots; artificially cured hay, cut green. Carotene-poor feeds—all hay that has lost its green color in curing; dry corn fodder (corn stover); straw, all kinds; corn, both yellow and white; wheat; oats; all by-product concentrates, such as beet-pulp, bran and oil meals.

It is seen from this list that there are relatively few feeds which furnish an abundance of carotene for butter. Of these, pasture grass, alfalfa hay and soiling crops are the only ones which cows in this country receive as their sole ration. Pasture and soiling crops are the summer feeds. The list shows that winter feeds in most cases are carotene-poor. Practically the only exceptions are properly cured hays. Although rich in carotene they can not be expected to give butter its summer color when they form only a part of the ration.

There is a rather striking difference among the various breeds of dairy cattle with respect to the amount of carotene incorporated in the butter fat. Guernsey and Jersey breeds rank first in this respect, with the Ayrshire, Shorthorn, Holstein and other breeds lower in the scale. Actual quantitative differences in carotene content of butter fat from representative animals of different breeds on the same kind of ration are not yet available, determined by acceptable, quantitative methods. The breed differences are relative, however, and at times disappear completely. For example, all breeds give very highly colored colostral milk, as already pointed out, and, when no carotene is present in the food, the milk fat of all breeds becomes colorless. The color of butter often persists longer on carotene-free feeds in the case of Guernsey and Jersey breeds, which is the cause of the belief, formerly held, that these breeds synthesize the pigment for the milk fat. The fact that the adipose tissue of these breeds is also highly colored with carotene has given rise to the idea that the body fat serves as a storage of pigment to be used when the ration lacks carotene. Direct physiological proof of this idea is lacking. Recent evidence suggests that a considerable storage of carotene may take place in the livers of cattle; possibly this is much greater in the case of Guernsey and Jersey cows than of the other dairy breeds. However, no difference has been observed [52] in the apparent concentration of carotene in the blood serum of different breeds on the same feeds. It must be that a much larger proportion of the carotene-carrying components of the blood is used in milk fat synthesis by Guernsey and Jersey cows than by those of the other breeds. A closer study of the carotene-carrying components of cattle blood from the different breeds seems warranted, as well as the determination of their possible relationship to milk fat synthesis.

Significance of color of milk and butter. The degree of yellow pigmentation of whole milk, and of butter to which no artificial coloring has been added, is determined primarily by the amount of carotene ingested and by the breed of cattle from which the milk and butter come. The breed influence on carotenoid pigmentation of milk is primarily evident

in the case of pure-bred or nearly pure-bred animals of the various breeds or herds composed solely of animals of such breeding.

The substantiation of the early finding of Steenbock [66] that carotene possesses vitamin A activity and the discovery [41] that this is due to the conversion of carotene to vitamin A in the animal body, probably in the liver, seemed to indicate that milk and butter richly pigmented with carotenoids by natural means should possess greatly enhanced nutritive value because of this pigmentation. Especially did it seem probable, when comparing milk and butter from the breeds having high and low carotenoid pigmentation on the same ration, that the milk from the highly pigmented breeds should show considerably higher vitamin A activity both because of its greater carotene content and also its greater fat content, and that the butter likewise should exhibit a correspondingly great vitamin A potency. These expectations have not been substantiated either for whole milk,[7] cream [22] or butter.[30, 70]

There appear to be at least three factors which contribute to the explanation of this apparently paradoxical situation. The first is that actual determinations [2, 19] of carotene and vitamin A concentrations by the most reliable methods available, in samples of butter fat representing widely different carotene concentrations resulting from variations in feed, show that the vitamin A content of the butter fat is apparently always considerably greater than the carotene content and varies considerably less. In one series [19] of 38 samples the carotene ranged from 0.0465 mg. to 0.703 mg. per 100 grams fat with a corresponding range in vitamin A from 0.168 mg. to 0.888 mg. In another series [2] of 14 samples the carotene range was 0.20 mg. to 0.86 mg. per 100 grams fat with a corresponding vitamin A range of 0.90 to 2.0 mg.

The second explanation rests on less secure grounds indicating that the total vitamin A activity of butter is due only in small part to the carotene present,[2, 61] namely, approximately three and one-half to seven per cent. The two studies referred to used different methods of estimating the vitamin A activity of carotene. In other series,[6,19] each using still different methods of calculating vitamin A potency of carotene, the carotene in butter fat was allowed a proportion of the total A activity ranging from 12.5 to 38.5 per cent. It may be pointed out that these higher values are attributable in part to methods of analysis involving either losses in true vitamin A content or certain assumptions of unproven certainty and thus are likely to be less accurate than the lower estimates. Moreover, the latter are in line with the experimental observations under discussion showing a lack of difference between vitamin A potency of milk, cream and butter from the different breeds of dairy cattle on the same ration.

The third explanation of this lack of difference is based on the suggestion [30] that breed differences in pigmentation represent a definite difference in ability to convert carotene to vitamin A.

It must be acknowledged that the comparisons of vitamin A potency of milk, cream and butter from the different breeds on the same ration

have not been concerned with simultaneous analysis for carotene and vitamin A. This should be done by the most reliable methods available. However, all of these more recent observations coincide with the earlier observations which showed that the feeding of cod liver oil [9, 10] to cows giving milk deficient in both vitamin A and carotenoids caused a great increase in vitamin A activity but no increase in pigmentation. Also it is now clear why yellow corn which has long been known [64, 65] to have marked vitamin A activity may be an important factor in contributing to the vitamin A content of butter although without appreciable effect on its natural pigmentation. The carotene present, which causes the vitamin A activity of the grain, is probably converted for the most part into true vitamin A in the cow.

Artificial butter color. One of the common yellow dyes used to artificially color butter is bixin, commonly called "annatto" or "orlean," from the seeds of annatto, *Bixa orellana*. The recent [27] elucidation of the chemistry of this dye places it with the carotenoids because it, like carotene, has an unsaturated aliphatic polyene structure. It has a number of chemical and physical properties in common with the true carotenoids, but does not possess vitamin A activity.[14] The vitamin A derived from carotene still contains the ionone ring which seems to be necessary for this biological property. Bixin contains no carbon rings, but instead is a dicarboxylic acid, $C_{23}H_{28}(COOH)_2$.

Water-Soluble Pigments

Historical. It is not definitely decided at the present time whether cow's milk contains more than one water-soluble pigment. Mention of the water-soluble coloring matter of milk appears in the scientific literature as early as 1784,[60] but the first attempt to isolate a definite substance responsible for the color was made nearly 100 years later [5] when the pigment isolated was given the name lactochrome. At one time [8] the greenish-yellow color of milk whey was regarded as caused by the urinary pigment urobilin, but this was later disproved.[4, 49] On the other hand, the so-called lactochrome has been shown to be closely related to urochrome, the more important of the urinary pigments. The name protochrome has also been suggested [56] for the whey pigment inasmuch as it has been shown that a substance with similar properties can be produced artificially from casein or peptone.

One of the outstanding characteristics of milk serum from cow's milk, as well as ultrafiltrates from milk, is the greenish-yellow fluorescence of the pigmented solution. Fresh whole milk, skim milk, buttermilk, as well as the greenish-yellow whey exhibit a strong yellow-green fluorescence under filtered ultra-violet [18] or a mercury vapor quartz lamp.[57] The yellow-green fluorescence changes to blue on reduction (loss of oxygen from the fluid) and may be restored by shaking in the air. Oxidizing agents destroy the yellow-green fluorescence, which is believed [57] to be

due to the lactochrome present in the dairy products exhibiting this property.

Recent investigations of the yellow pigments having a yellow-green fluorescence and occurring in various organs and tissues of animals [11] as well as in materials from plant sources have led to renewed interest in the whey pigment and to the proposal that all these yellow pigments having yellow-green fluorescence be given the class name lyochrome,[11] and that the representative (or representatives) of this class of pigments in whey be called lactoflavin (or lactoflavins).

According to Bleyer and Kallmann [4] the lactochrome first isolated by Blyth [5] and later studied by Palmer and Cooledge [50] in relation to urochrome is not the true whey pigment but an artificial product. Their most highly purified preparation, however, was admittedly not the pure whey pigment. It seems probable that the isolation of the pure crystalline substance has now been accomplished,[34] although, as previously stated, the possibility exists [12, 13] that more than one representative of the lyochrome class of pigments occurs simultaneously in cow's milk.

Preparation of lactoflavin (lactochrome). One of the principal problems in the preparation of the whey pigment is in securing suitable concentrates, free from protein and lactose. Various methods have been proposed for accomplishing this. Blyth removed the casein by acid, the albumin and globulin by heat coagulation, and then precipitated the pigment by addition of acid mercuric nitrate, which was filtered off, suspended in water and decomposed with H_2S. The efficacy of this procedure was confirmed by Palmer and Cooledge. The latter showed that the whey pigment behaves like urochrome in being extracted by shaking the heat-coagulated, filtered whey, which has been saturated with $(NH_4)_2SO_4$, with 98 per cent ethanol, the alcohol layer which rises containing all the pigment. Bleyer and Kallmann applied this procedure to concentrates from heat-coagulated whey. Radley adds an excess of 95 per cent ethanol to the whey, centrifuges off the precipitate, concentrates the filtrate, repeats the extraction and concentration of the filtrate twice more, the final alcohol extraction being with 80 per cent ethanol. The water-soluble material from the residue left after evaporation of the last alcohol extract contains the pigment concentrate.

The newer methods, which have led to the isolation of pure pigment substance, make use of the adsorption by fullers' earth to accomplish the first concentration of pigment. Acid whey or rennet whey acidified to pH 4 to 5 is treated with 1 part fullers' earth to 50 to 100 parts whey. After a suitable period (1 to 24 hrs.) the fullers' earth is recovered by centrifuging or settling and is washed either with distilled water (free from chlorides) or distilled water followed by ethanol. Elution of the pigment is accomplished either (a)[34] by a mixture of equal parts of pyridine and methanol, diluted with two parts water or (b)[12] by 80 per cent pyridine-water solution acidified with 0.83 part glacial acetic acid. Kuhn and associates employ one elution with about seven volumes of diluted pyridine-methanol for each kilogram of fullers' earth used. Ellin-

ger repeats the elution four to six times with six volumes of elution fluid per kilogram of fullers' earth employed. The extracts in either case, when concentrated in vacuum and purified from suspended matter and lipids, represent crude solutions of the whey coloring matter.

Final purification of the concentrated solutions was attempted by Bleyer and Kallmann by the following methods. (1) All concentrated preparations were first carefully evaporated in vacuum to a thick syrup. The syrup was then extracted twice with four volumes of a mixture of 2 parts chloroform and 1 part 96 per cent ethanol by shaking for one-half hour with warming on a water bath. The combined extracts were evaporated again in vacuum and the extraction repeated using this time 8 volumes of a mixture of 8 parts chloroform and 1 part ethanol. This extract was evaporated in a vacuum desiccator over a mixture of fused and liquid paraffin to absorb the chloroform. (2) The pigment was adsorbed from its concentrated aqueous solutions by means of colloidal aluminum hydroxide gel, from which it could be extracted by ethanol or NH_4OH or ammoniacal ethanol. (3) The pigment was precipitated from its concentrated aqueous solutions by lead acetate in the presence of a large excess of NH_4OH. The lead compound was decomposed with H_2SO_4 and the excess acid removed by $Ba(OH)_2$ in the usual manner.

Crystalline lactoflavin may be isolated as follows.[34] (The papers of Ellinger and Koshara should also be consulted.) The concentrated elution fluid is freed from colloidal fullers' earth by adding one to two volumes of methanol. The centrifuged solution is freed from methanol in vacuum; lipids are removed by repeated ether extraction and finally by addition of 10 volumes of acetone. The pigment is now readsorbed, the adsorbent washed and elution effected with a slightly diluted pyridine-methanol mixture (75:200). This elution is concentrated under reduced pressure and treated with a large volume of acidified acetone (using acetic acid). After centrifuging out the precipitated impurities the solution is concentrated again in vacuum to very small volume, filtering off colorless impurities which come down in the course of the concentration. Creatinine and other impurities are now precipitated with picric acid, the picric acid removed by ether in the dark, and the pigment allowed to crystallize out of the reconcentrated solution. Recrystallization is secured from boiling 2*N* acetic acid. Six mg. were obtained from 300 liters of milk. The crystals are brown-orange fine needles, darkening at 240°, decomposing with sintering at 267° (271° corrected).

Constitution and properties. The lactochrome first isolated by Blyth contained nitrogen, as did the crude lactochrome of Bleyer and Kallmann. Lactoflavin is also a nitrogenous substance probably having the empirical formula $C_{17}H_{20}N_4O_6$.[34] ($C_{16}H_{20}N_4O_6$ has also been suggested.[13]) The molecular structure has not yet been determined. Kuhn and Wagner-Jauregg[36] state that the available evidence points to a heterocyclic structure, one ring containing two N atoms as —NHCONH—, a second ring rich in ester-forming hydroxyls, and a third ring contain-

ing two tertiary N atoms concerned with the color-forming unsaturated carbons.

Pure lactoflavin dissolves readily only in water to give yellow to orange solutions having an intense green fluorescence. It is also soluble in dilute ethanol and methanol, in mixtures of chloroform and ethanol and in pyridine, acetone and glacial acetic acid. Aqueous and alcoholic solutions are decolorized by alkalis and by light, the latter also forming a colored decomposition product called lumiflavin. Solutions in pyridine or glacial acetic acid are much more light stable. Lactoflavin is very stable to acids and to certain oxidizing agents (Br_2, HNO_3, HNO_2) but not to chromic acid or permanganate. The pigment forms insoluble compounds with the heavy and noble metals and with phosphotungstic and phosphomolybdic acids, the latter two compounds being soluble in dilute acids. The green fluorescence of aqueous solutions is a function of the pH value, disappearing on addition of either acids or alkalis. Alkaline solutions which have lost their fluorescence, retain their color, but this is destroyed by sodium hyposulfite. On addition of H_2SO_4 to this solution the color is restored and, on neutralizing with sodium acetate, also the green fluorescence.

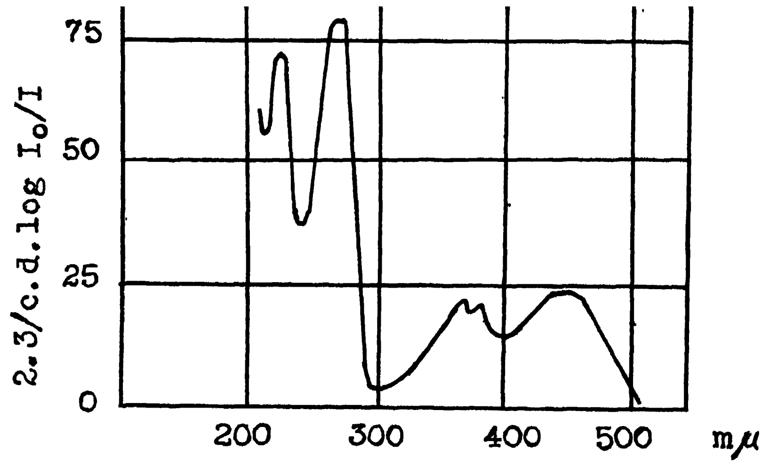


FIG. 4.—Spectrophotometric transmittancy of aqueous solution of lactoflavin, according to Kuhn, György and Wagner-Jauregg.[34]

Abscissa: Wave lengths in mμ. Ordinates: molecular extinction coefficient × 10^{-3}; molecular weight assumed = 364. (c in mols per liter, d in cm.)

Lactoflavin forms compounds with saccharides, proteins and purines (uric acid). These compounds possibly either occur naturally in milk or readily form during isolation, thus accounting for the several lactoflavins[12] isolated from milk. Acetyl esters are formed on treatment with pyridine acetic anhydride, soluble in chloroform; these retain the original color and fluorescence of the uncombined pigment. An acetate

is crystallizable, forming orange-colored, matted needles, darkening at 220° and melting at 243°, with decomposition. The acetates are hydrolyzed by soda, regenerating the pigment.

The earlier investigators of lactochrome observed no visible spectroscopic properties. However, Palmer and Cooledge[50] found that "active" acetaldehyde[16, 17] and heat caused the appearance of a visible band at the F (486.2 mμ) line and subsequently a second less prominent band farther towards the blue, the color of the solution deepening to orange. Pure lactoflavin and its acetyl ester examined spectrophotometrically show the spectroscopic transmittancy illustrated in Figure 4.[34] The three bands in the visible spectrum correspond roughly with the two bands seen by Palmer and Cooledge on treatment of crude lactochrome solution with "active" acetaldehyde, the two shallow peaks between 350 mμ and 400 mμ appearing to them as a single band. The nature of "active" acetaldehyde has not heretofore been known. It now seems probable that it contains acetic anhydride and that the acetyl ester of lactochrome (lactoflavin) more readily exhibits the absorption maxima in an ordinary narrow dispersion spectroscope. The five maxima for pure lactoflavin acetate are at 445 mμ, 380 mμ, 365 mμ, 265 mμ and 220 mμ, the latter two being in the ultra-violet.

Significance of lactoflavin. The yellow whey pigment is regarded[18, 36] as being related to the yellow co-enzyme, obtainable from heart muscle,[1] red blood cells[68] and yeast.[68, 69] It is the oxidized form of the substance concerned with oxidation-reduction phenomena in which it functions. Of more direct significance is the probability[21] that the pigment is vitamin B_2 (G), in which milk is now known to be relatively rich. A daily dose of 5 γ (0.005 mg.) of thrice recrystallized lactoflavin given to rats suffering from deficiency of vitamin B_2 (G) is sufficient to stimulate weight increase of 35 grams to 75 grams in four weeks. Palmer and Cooledge[49] observed a more intense yellow color of whey when cows were fed alfalfa hay and grain than when they received bleached clover or timothy hay and grain. Hunt and Krauss[24] found that milk from cows on pasture has a higher vitamin G content than milk from cows on dry feed with the possibility that the quality of hay fed in stall-feeding may also be a determining factor. These observations regarding variations in whey pigmentation and vitamin G activity of milk seem significant in the light of the vitamin B_2 (G) action of lactoflavin. The possibility of the analysis of milk and other foods for vitamin G, using spectrophotometric methods, would appear to be realizable.

A significant relation of breed to milk whey color has been observed. A study[49] of the color of the whey of 43 cows comprising 4 Ayrshires, 4 Shorthorns, 15 Holsteins and 20 Jerseys, varying in age from 3 to 15 years, with a milk production ranging from 4.2 to 47.4 lbs. daily, in various stages of the lactation period extending from 3 to 13 months gave the following results:

Breed	Units of Yellow
Ayrshire	4.78
Jersey	3.49
Holstein	2.41
Shorthorn	2.15

(The units of yellow represent color of a depth of 10 cm. of clear whey as matched by the Lovibond tintometer glasses.)

Lactochrome apparently is not confined to cow's milk. The milk of other species also contains a greenish-yellow whey pigment. This is true of human milk. The whey of ewe's milk contains as much color as cow's milk and at times a much higher concentration. The milk of other species has not been examined. Conclusive proof that the whey pigment of other species is identical with lactochrome is lacking, but it is reasonable to suppose that such an identity exists.

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Chapter V

Lactose

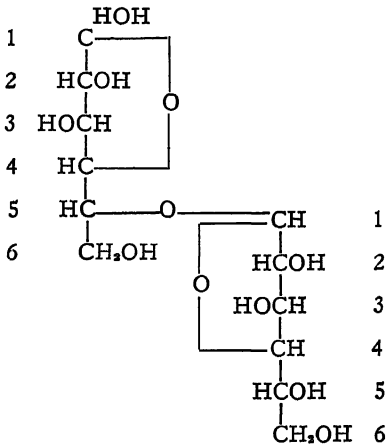
Occurrence. Lactose, or milk sugar, $C_{12}H_{22}O_{11}$, is known to occur in the milk of all land mammals and probably is present in the milk of all mammals, although the evidence is conflicting for the milk of the whale. The amounts in the milks of different mammals vary from about 2.0 per cent to about 8.5 per cent. The lactose percentage in cow's milk has been found to vary from 2.7 to 5.5, 80 per cent of cows giving milk containing from 4.0 to 5.0 per cent sugar.[82] Human milk contains a much higher percentage of lactose than does cow's milk; hence the practice of adding lactose to cow's milk for infant feeding. No other saccharide occurs to any appreciable extent in milk, nor does lactose occur elsewhere, unless uncorroborated reports of its isolation from two fruits may be accepted.[90]

Constitution. Lactose is a disaccharide yielding on hydrolysis *d*-glucose and *d*-galactose. Mild oxidation of lactose yields a monobasic acid, this indicating that only one of the two aldehyde groups of the constituent monosaccharides is free to function in lactose. The fact that the hydrolysis of any simple derivative of lactose invariably gives galactose and the corresponding glucose derivative proves that the active aldehyde group of lactose is the aldehyde group of the glucose end of the molecule. Since the aldehyde group of galactose does not exist as such in lactose, the carbon atom of this group must be the point of union of the galactose residue to the glucose residue and lactose must be a glucose galactoside. The carbon atom of the glucose linkage was not definitely identified till fairly recently. Haworth and Leitch[27] methylated lactose and hydrolyzed the resulting heptamethyl methyllactoside. They obtained 2-3-5-6-tetramethyl galactose, as was to be expected on the assumption (at that time considered justified) of a 1-4-lactone structure for the hexoses, and 2-3-6-trimethyl glucose. Since the 1 group of the glucose residue was known to function as an aldehyde group in lactose and the 4-carbon atom was assumed to be involved in the lactone ring, the 5-carbon atom was left to be accounted for by the disaccharide linkage. Formula I was offered on the basis of these results.

Later, Charlton, Haworth and Peat[5] showed that hexoses exist in nature in the form of amylene oxides, or 1-5-lactones. On the assumption that the amylene oxide linkages persist in disaccharides and on apparently satisfactory evidence that what had been thought to be 2-3-5-6-tetramethyl galactose was really 2-3-4-6-tetramethyl galactose, they revised the formula of lactose to II. At about the same time conclusive evidence that the 4-carbon atom is the point of linkage of the glucose was obtained by Zem-

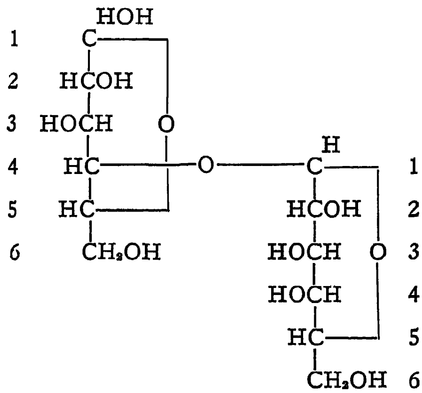
plén,[95] Levene and Sobotka[52] and Haworth and Long,[28] each group of workers using a different technic.

Hudson,[41] reasoning that the molecular rotational difference between β-lactose and β-cellobiose must be the same as that between an α-methyl galactoside and an α-methyl glucoside having the same lactone rings respectively, compared the molecular differences of the four possible pairs made up from 1-4-α methyl galactoside and 1-5-α methyl galactoside on the one hand and 1-4-α methyl glucoside and 1-5-α methyl glucoside on the other with that of the β-lactose β-cellobiose pair. Since the agreement was with the difference between the 1-4-galactoside and the 1-5-glucoside, these lactone structures were allocated to the secondary hexose groups of lactose



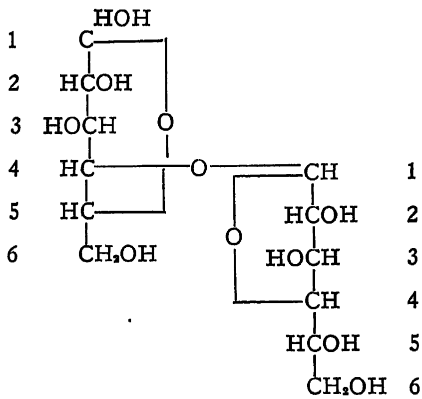
Glucose residue. Galactose residue.

I.



Glucose residue. Galactose residue.

II.



Glucose residue. Galactose residue.

III.

and cellobiose respectively. Hence, formula III seems at present to be the best authenticated structural arrangement for lactose. These formulas do not show the α-β asymmetry of lactose, which depends on the arrangement of groups on the 1-carbon of the glucose end of the molecule.

Physical forms and equilibrium relationships. Lactose may be prepared in three homogeneous forms, two being anhydrous, the other a monohydrate.[25, 34, 35, 36, 38, 79] Ordinary commercial lactose is the monohydrate and may be prepared by crystallizing from water solution at temperatures below 93.5°. The other forms change to the hydrate below 93.5° in the presence of small amounts of water, this indicating the hydrate to be the stable solid form at ordinary temperatures. The hydrate has a specific rotation of $[\alpha]^{D}_{20°} = +89.4°$ and a melting point of 201.6°.[90]

If the crystallization takes place above 93.5°, the crystals are anhydrous, have a specific rotation of $+35.0°$ and a melting point of 252.2°. If the more nearly perfect crystals are selected and washed successively with hot glycerine, hot 95 per cent ethyl alcohol, and ether, a product of a high degree of purity is obtained. That this is the stable solid form above 93.5° is proven by its method of preparation and the fact that both other solid forms change to this form above 93.5° in the presence of small amounts of water.

The third form may be prepared by dehydrating the hydrate at any convenient temperature above 65° under vacuum. This form may be preserved indefinitely in the absence of water, but in the presence of small amounts of water it changes to the other anhydride above 93.5°, to the hydrate below 93.5°. Its melting point is 222.8°.

Since the aldehyde group of the glucose end of the molecule must be the group concerned in the rotational differences between the two forms,—the aldehyde group of the galactose end being non-functional,—the distinguishing Greek letters are assigned to the anhydrous forms of lactose on the basis of their analogy to α- and β-glucose. β-lactose then is the form having the specific rotation of $+35.0°$, and the metastable form is α-lactose. α-Lactose has been shown to be α-glucose β-galactoside, β-lactose to be β-glucose β-galactoside.[15, 16]

Since the decomposition of lactose begins at 130°, it has not been possible to determine the transition point for α-anhydride $\rightleftarrows$ β-anhydride. The determination of the melting points by the quick method of Soch has shown, incidentally, that the transition point is above the melting points. The transition point β-anhydride $\rightleftarrows$ hydrate is known to lie between 93.3° and 93.8° and is usually considered to be 93.5°. Gillis reasons that, since the α-anhydride is always formed on dehydration of the solid hydrate, the hydrate must be of the α form and that 93.5° must be both a transition and a dehydration point. The fact that no solid β-hydrate has been isolated he takes to prove that its solubility is greater than that of the β-anhydride.

The values for specific rotations given above are initial values. After 24 hours standing at room temperature, a solution that has been prepared from any form of lactose shows a specific rotation $[\alpha]^{20°}_{D} = +55.5°$. This change in rotation is gradual and is the phenomenon known as mutarota-

tion. Its rate is considerably affected by changes in temperature and in pH value of the solution. At 0° an equilibrated solution contains 62.25 per cent of its lactose in the β form and 37.75 per cent in the α form. The equilibrium constant is therefore 62.25/37.75 or 1.65. This value is slightly affected by changes in temperature, but not by those in pH value.[63]

Parisi[63] has investigated in considerable detail the effect of different pH values on the rate of change in solution of β- to α-lactose. He found the values of k_2, the velocity constant, to depend on temperature, H-ion concentration and OH-ion concentration, and derived the equation, $k_2 = ax^{pH} + by^{pOH}$, in which a and b are coefficients depending on the temperature, and x and y represent the "rates" of the two geometric progressions involved. The value of k_2 is at a minimum at about pH 5.0, and the effects of the H-ion concentration and the OH-ion concentration are equal at about pH 7.0. If k_2 is plotted as ordinate against pH value as abscissa, the curve is not symmetrical to a vertical line at approximately pH 5.0, as frequently assumed, but rises more sharply on the alkaline side than on the acid. Parisi found the logarithm of the "rate" for the geometric progression as a function of OH-ion concentration to be seven times that of the logarithm of the corresponding "rate" for the progression as a function of H-ion concentration.

We may now consider the formulation of the conditions in a lactose solution. According to an earlier view, the equilibrium relation was the following:

$$\alpha\text{-anhydride} + H_2O \underset{1}{\rightleftarrows} \text{hydrate} \underset{2}{\rightleftarrows} \beta\text{-anhydride} + H_2O.$$

According to this formulation, equilibrium 1 must be quickly established and equilibrium 2 slowly established. But the reverse statement must be made for the otherwise analogous equilibria for maltose. Gillis objected to this theory on account of this difficulty and on account of the assumption of $-CH(OH)_2$ as the terminal group of lactose hydrate, which denies the lactonic structure of the hydrate and the asymmetry of its terminal carbon atom. Hudson's work has shown that the rotational effect of the terminal group of aldoses has a definite additive value which is positive or negative depending upon whether the aldose is of the α or β form. In the case of lactose, it would seem to follow from the data that the terminal group of the hydrate is the mirror image of that of the β-anhydride and is identical with that of the α-anhydride. Gillis formulates the equilibria thus:

$$\begin{array}{ccc} \alpha\text{-anhydride } (+ H_2O) & \rightleftarrows & \alpha\text{-hydrate} \\ \uparrow\downarrow & & \uparrow\downarrow \\ \beta\text{-anhydride } (+ H_2O) & \rightleftarrows & \beta\text{-hydrate} \end{array}$$

The hydration equilibria become established almost instantaneously; the α-β equilibria become established more slowly and are the real basis of the mutarotation phenomenon. This theory does not require the assumption of speeds of different orders for analogous reactions of the same sugar or of different sugars.

Solubility. Lactose hydrate is insoluble in 95 per cent ethanol, methanol and ether, soluble to about 2 per cent in pyridine, and somewhat soluble in warm acetic acid, either concentrated or dilute, from which it crystallizes unchanged on cooling. It dissolves in about 6 parts of cold or 2.5 parts of hot water and crystallizes therefrom as rhombic prisms. It easily forms supersaturated solutions in water. In nearly saturated sucrose solution, the solubility of lactose is about one-half its value in water alone.[64] The taste of lactose hydrate is about one-sixth as sweet as that of sucrose and, on account of the low solubility and hardness of the crystals, it produces on the tongue a sensation like that produced by sand. The solubility and freezing point relationships of lactose are of fundamental importance in the consideration of "sandy" ice cream, since it has been proven that lactose crystallization is the cause of "sandiness."

Hudson has worked out the solubility relationships of lactose in water at various temperatures. Table LVIII is based on his data. The first column shows the solubility of the hydrate, or the initial solubility; the other the solubility of the equilibrium mixture, or the final solubility. The β form is exceedingly soluble,—about 45 parts to 100 parts water at 0° and about 95 parts to 100 at 100°. Its solubility is not of great interest in the dairy industry, unless, of course, this form should be found to be a factor in the "sandiness" problem under conditions where the freezing out of water as ice gives rise to very highly concentrated sugar solutions.

Table LVIII.—Initial and final solubility of lactose.

Initial			Final		
Temperature	Lactose	Parts of lactose to 100 of water	Temperature	Lactose	Parts of lactose to 100 of water
°C.	per cent		°C.	per cent	
0	4.7	5.0	0	10.6	11.9
15	6.6	7.1	15	14.5	16.9
25	7.9	8.6	25	17.8	21.6
39	11.1	12.6	39	24.0	31.5
49	15.1	17.8	49	29.8	42.4
64	20.7	26.2	64	39.7	65.8
74	25.6	34.4	74	46.3	86.2
89	35.8	55.7	89	58.2	139.2

Crystallization. If a lactose solution is cooled until the saturation point is passed, and crystallization starts, it is the hydrate which separates out. If the physical conditions of the solution are favorable, this separation will continue with considerable rapidity until the amount of hydrate in excess of the quantity soluble at this temperature is removed. But, at the moment some of the hydrate separates, a portion of the β-lactose in solution changes to the hydrate form to re-establish equilibrium. Since the solution is already saturated with hydrate, further separation of hydrate will occur. Since this transformation rate at low temperatures is

markedly slower than the crystallization rate, the speed with which this reaction takes place controls the rate of further lactose separation. The transformation rate is in turn quite sensitive to relative hydrogen-ion and hydroxyl-ion concentrations and to temperature. The relative percentages of lactose transformed in one hour were found by Hudson to be—

At 25°	51.1 per cent
At 15°	17.5 per cent
At 0°	3.4 per cent

The reaction is practically instantaneous at 70°.

The rate of crystallization of lactose from its supersaturated solutions at various temperatures from − 5° to + 30° was studied by Whittier and Gould,[92] who found that, for the first two and one-half hours, the rate of separation was greater at 30° than at 25° or any lower temperature, as shown in Figure 5. The subsequent decrease in rate of crystallization at 30° is a result of the considerable decrease in the concentration of the lactose in solution. Not until about seven hours have elapsed does the amount of lactose which separates at 25° approach the amount which separates at 30°. From this it appears that the most efficient method of crystallizing lactose is to cool the solution fairly rapidly to approximately 30°, to hold it at that temperature for about 3 hours, then to cool it to near 20°, and to hold it at that temperature for as long as otherwise practical before filtering out the crystals. There is no practical advantage in the use of ice-box temperatures, but rather a distinct disadvantage so far as efficiency of crystallization is concerned.

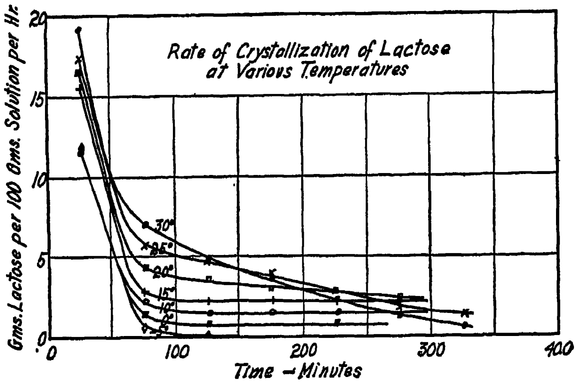


FIG. 5.—Rate of crystallization of lactose from solutions at various temperatures.

Another point of considerable interest is the fact that supersaturated solutions of lactose exhibit extensive labile zones.[51] This means that lactose solutions may be highly supersaturated but may not crystallize, even when agitated, unless lactose crystals or particles of some amorphous substance are present. Even then a spontaneous separation may not

occur, the nuclei alone growing. Of course a sufficiently high degree of supersaturation will cause spontaneous separation to occur. The boundary line of this zone, together with the solubility curves based upon Hudson's data, is plotted in Figure 6.

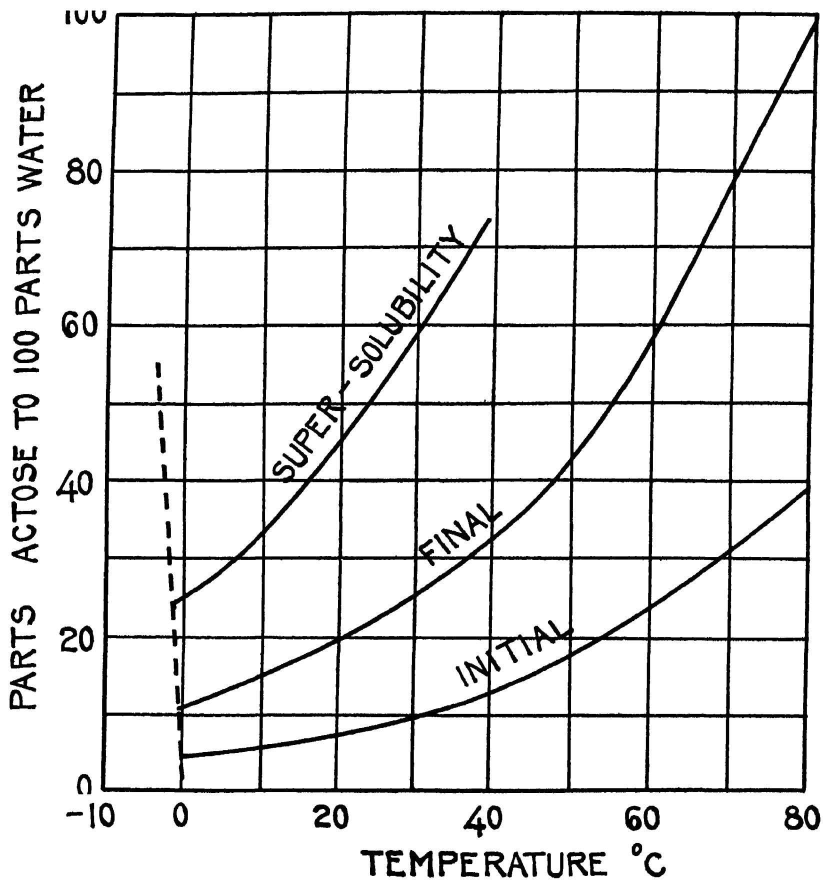


FIG. 6.—Solubility of lactose in water at various temperatures.

Lactose has measurable acidic properties, binding at least two mols NaOH per mol sugar. Its first two apparent acid dissociation constants have been determined[84] and found to be 1.20×10^{-12} and 3.63×10^{-14}.

Other physical constants. The coefficient of cubical expansion of lactose is 0.00911 per degree between 0° and 100°.[43] Values given for the heat of combustion of lactose vary from 3663 to 3953 calories per gram for the hydrate, and from 3737 to 4162 calories per gram for the β-anhydride.[90] The heat of formation of the hydrate is 535.6 Calories per mol, of the β-anhydride 610.8 Calories per mol.[81] The specific heat of the hydrate

is 0.299, of the β-anhydride 0.2895.[55] Table LIX gives values for heats of solution and transition of the different forms of lactose.[87]

Table LIX.—Heat of solution and transition of lactose. 20° C.

	Hydrate	α-anhydride	β-anhydride
	cal/gram	cal/gram	cal/gram
Initial heat of solution	−12.	+7.3	−2.3
Final heat of solution	−11.4	+7.9	−2.7
Temp. coef. of heat of solution	0.1	....	....
Heat of transition to β-anhydride	+1.0	+1.0	....
Heat of transition to equilibrium mixture	....	+1.3	....

The axial ratio of the crystals of the hydrate is given variously[72, 88] as $a:b:c = 1 : 0.6215 : 0.2193$ and $a:b:c = 1 : 0.3677 : 0.2143$, that of the β-anhydride as $a:b:c = 0.817 : 1 : 0.377$.[89]

The specific gravity of the hydrate has been determined with a high degree of accuracy and found to be 1.5453. The specific gravity of the β-anhydride is 1.59. Fleischmann and Wiegner,[23] and Schmoeger[73] have determined the changes with concentration of the specific gravity and contraction of lactose solutions. The maximum contraction is 0.593 cc. per 100 grams of solution and occurs at a concentration of 54.03 per cent. Such a solution contains 18 mols H_2O for each mol of $C_{12}H_{22}O_{11}$.

Hydrolysis. Lactose may be hydrolyzed by the lactase of the intestinal wall of mammals, by the lactase present in almonds and in certain yeasts such as *Saccharomyces tyrocola* and the *Saccharomyces kephir* of kephir grains, and by dilute strong acids. The production of alcohol from lactose by yeast is possible only when the yeast contains lactase. Hydrolysis precedes the alcoholic fermentation in this case. Glucose and galactose in equal quantities are the immediate products of hydrolysis; the formation of two molecules of glucose from one molecule of lactose in the intestine is undoubtedly due to a conversion of galactose to glucose after the hydrolysis. A number of weak acids—e.g., citric—which easily hydrolyze sucrose are unable to hydrolyze lactose under the same conditions. For the complete inversion of the lactose in a five per cent solution, it is recommended that ten cc. of 36 per cent hydrochloric acid be added for each 100 cc. and that the solution be held at 90° for 90 minutes.[90]

Synthesis. Lactose has been synthesized from β-galactose and β-glucose by heating under reduced pressure in the presence of zinc chloride.[66, 67] The synthetic compound was shown to have the same characteristic properties as the lactose from milk.

Related sugars. Two sugars, gynolactose and allolactose, have been reported as isolated from human milk.[68] The characteristics of gynolactose have not been determined, but allolactose is perhaps identical with the 6-galactosido glucose prepared by Helferich and his coworkers.[30, 31] Lactulose[59] is a sugar artificially prepared by conversion of lactose to the

corresponding keto sugar. It is 4-β-*d*-galactosido(1,4)-*d*-glucose and, in the crystalline form, is an α sugar.

By shaking finely powdered lactose in methanol containing dry HCl, there has been prepared what is evidently a molecular compound containing 5 molecules of anhydrous α-lactose to 3 molecules of anhydrous β-lactose.[38] The method of preparation suggests the possibility that other molecular compounds of this type may be prepared by proper choice of medium.

Oxidation. A great number of different substances may be obtained from lactose by oxidation with different oxidizing agents and under various physical conditions. Since investigations on sugar oxidation so far reported in the literature have been carried out mostly under purely empirical conditions with several factors of prime importance uncontrolled or unmeasured, it seems worth while to list only a few of the oxidants and their corresponding products. Bromine oxidizes the terminal aldehyde group of lactose to carboxyl, forming lactobionic acid, $C_{12}H_{22}O_{12}$. If the solution is unbuffered, hydrolysis and further oxidation by the same reagent give galactonic and gluconic acids. Oxidation by bromine water of α-lactose buffered at pH 6.0 is approximately one-half as fast as that of the β form under the same conditions.[42] Formic, acetic and various saccharinic acids are formed by air oxidation of lactose in alkaline solution.[10, 46, 47, 48, 49, 61] The autoxidation of lactose in the presence of dilute acid yields, among other substances, levulinic and formic acids. This is probably one of the changes which take place when milk is heated, since it has been shown that the source of the acid produced under such conditions is lactose and that the acid is partly formic.[91] Oxidation of lactose by concentrated nitric acid yields oxalic and carbonic acids. With nitric acid of 25 to 30 per cent concentration, there are obtained mucic and saccharic acids,—the 1,6-dicarboxylic acids derived from galactose and glucose respectively. Racemic and tartaric acids are also formed in small amounts by further oxidation of mucic and saccharic acids. Formic acid is formed by the action of dilute potassium permanganate in acid solution or by autoxidation of lactose in the presence of dilute acid.

Reduction. Hydrogenation in the presence of nickel gives the three sugar alcohols, lactositol, dulcitol and sorbitol.[76] The aldehyde groups of the lactose and of the galactose and glucose formed by hydrolysis become primary alcohol groups. The action of sodium amalgam on lactose produces dulcitol, sorbitol, and smaller amounts of simpler substances.[4]

Effects of heat. Lactose hydrate may be heated to 110° without change, but between 110° and 130° it loses all its water of crystallization.[53] The anhydrous lactose becomes yellow in color at 150° to 160° with no perceptible change in weight. At 175° it becomes brown, emitting a characteristic odor, and losing about 13 per cent of its original weight. If the heating is not carried above this last temperature, a mass is obtained containing some anhydrous lactose, lactocaramel, and a substance which is insoluble in water. The first may be removed by means of ethanol and the lactocaramel then dissolved in water. This substance has the empiri-

cal formula $C_{12}H_{20}O_{10}$. By dehydration of lactose at 185° for 10 to 12 hours under 4 to 6 mm. pressure, there is obtained what is apparently the same compound.[65] The name lactosan has been applied to this substance, since constitutionally it is probably 5-galactosyl glucosan. It polymerizes in the presence of $ZnCl_2$ at 105°. Pyrocatechol and succinic acid have been reported as products of the action of heat on lactose in the presence of alkalis.

Lactose phenylosazone. The formation of the phenylosazone is one of the most widely used methods of identifying sugars.[12] In the case of lactose, oxidation by nitric acid to give the sparingly soluble mucic acid distinguishes it from all other common sugars except galactose. However the formation of the osazone is useful as a confirmatory test. It is made by heating together 1 part lactose, 1½ parts phenylhydrazine hydrochloride, 2 parts sodium acetate and 30 parts water. As the solution cools, the osazone separates out as fine needles which melt at 200°. Since the melting point does not distinguish it sharply from the osazones of galactose and glucose, resort may be had to microscopic examination of the lactosazone or to the polarimetric examination of a solution of 0.2 gram in 4 cc. pyridine and 6 cc. ethanol. The rotation should be zero. Dehydration of lactose phenylosazone by means of 20 per cent H_2SO_4 yields an anhydride which melts at 223° to 224° (uncorr.).

Lactose nitrates. The literature lists tri-, tetra-, penta-, hexa-, and octo-nitrates as resulting from the action of nitrating mixtures on lactose.[80] The octonitrate, melting at 145° to 146°, is the most authentic.[93] This compound has the advantage over the nitrates of other common sugars that it can easily be stabilized, and consequently it has been used successfully both as a detonator and as a blasting charge.[6]

Other derivatives of lactose. Although none of the other derivatives of lactose so far prepared has any general importance at present, it seems desirable to list these derivatives here by name with citation in order that anyone interested in some specific compound may readily trace its literature. Compounds with a non-nitrogenous substituent on the aldehyde group are designated as lactosides with the name of the terminal substituent adjacent to the generic name.

Lactose phenylhydrazone [18]
Lactose amylphenylhydrazone [85]
Lactose allylphenylhydrazone [85]
Lactose benzylphenylhydrazone [85]
Lactose β-naphthylphenylhydrazone [85]
Heptaacetyl acetyllactoside [7, 32, 39, 75]
Heptaacetyl lactose [40]
Heptaacetyl bromolactoside [8, 19]
Heptaacetyl chlorolactoside [8, 17]
Heptaacetyl fluorolactoside [30]
Heptaacetyl methyllactoside [7]
Heptaacetyl thiophenollactoside [18, 70]
Heptaacetyl menthyllactoside [20, 24]
Heptaacetyl glycollactoside [24]
Heptaacetyl succinimidelactoside [24]
Heptaacetyl theophyllenelactoside [24]
Heptamethyl methyllactoside [27]
Methyllactoside [7]
Acetyl iodolactoside [58]
Butyryllactoside [2]
Fluorolactoside [30]
Thiophenollactoside [18, 70]
Menthyllactoside [20, 24]
Acetyl cyanolactoside [24]
Acetyl thiocyanolactoside [24]
Hexacetyl lactals [21, 22]
Pentaacetyl lactal [21, 22]
Lactals [21, 22]
Heptabenzoyl lactose [62]
Hexabenzoyl lactose [78]
Lactose anilide [45]
Lactose toluide [45]
Lactose ammonia [54, 86]
Lactose hexamethylenetetramine [50]

Lactose pyridide [69]
Lactose aminoguanidide [94]
Lactose ureide [74]
Lactose semicarbazone [56]
Lactose octophenylurethane [57]
Lactose cyanhydrin [14, 71]
Lactose carboxylic acid [14, 71]

Manufacture of lactose. Until comparatively recent years the manufacture of lactose has been carried out usually in connection with cheese manufacture, since whey, which is the source of lactose, is a by-product of the cheese industry and is too perishable and bulky to be shipped. Up to about 1880, lactose was produced only in Canton Luzerne, Switzerland. Shortly after this, factories were established in Germany and in the United States, and at the present time it is produced in other countries of Europe, and in South America, New Zealand and Australia. In 1914, the United States produced 3,500,000 pounds; in 1922, 2,190,000 pounds; in 1932, 3,700,000 pounds.

The original Swiss method was to prepare a "sugar sand" by a crude method of evaporation and crystallization at the small cheese factories high in the Alps. This "sugar sand" was brought to a central point for refining.

The modern methods for the manufacture of lactose use to a considerable extent equipment of the kind used by the cane and beet sugar industries. Lactose solutions are especially susceptible to the action of heat, and the use of a vacuum pan for evaporation is of great advantage in obtaining a white sugar.

The details of the treatment of raw whey depend on the type of whey used. Since most of the lactose produced in the United States is now made from the hydrochloric acid whey from casein manufacture, the method for handling that type of whey is described here.[60]

The whey is heated nearly to boiling in iron tanks, direct steam being used. During the heating, milk of lime is gradually added until the reaction is at about pH 6.2. When the heating is stopped, the albumin and calcium phosphate separate in a compact layer and the clear whey is drawn into a tank which is a reservoir to the vacuum pan. The albuminous layer is dropped into a sludge tank and filtered later.

The clear, partially neutralized and partially refined whey is evaporated to a thin syrup (about 20° Bé.) in a double effect vacuum pan and filtered in a filter press, which is then used to filter the albuminous sludge. The syrup is decolorized by means of carbon in some plants previous to this filtration. The press cake is broken up, dried and used in poultry feeds. The combined filtrates are evaporated further in a single effect pan to about 40° Bé., at which concentration crystallization commences. Hydrochloric acid may be added during this evaporation to prevent foaming. The hot syrup is run into jacketed crystallizers in which it is cooled and stirred, and, when crystallization appears to be complete, the mush of crystals is filtered in a centrifuge and washed sparingly with cold water. The mother liquor and wash water may be concentrated and a second crop of crystals obtained. The mother liquor from this second crop

usually contains so much salt that it is not practicable to recover more of the sugar and consequently this is discarded.

The above method has been modified in such a way that a high grade technical lactose may be made by means of only one crystallization.[88] After the protein-calcium phosphate precipitate is removed from the hot whey at a reaction of pH 6.2, the clear liquid is held at 50° and one part of trypsin added for each 10,000 parts of whey. Digestion is allowed to proceed for an hour, during which time the fractions of non-heat-coagulable protein remaining in the whey are broken down into smaller, more soluble units, which are easily washed from the crystalline sugar later on. From this point, the manufacturing procedure previously described is followed. Lactose made by this method is unusually free from protein and, consequently, its saturated solutions will not foam appreciably on boiling.

Lactose is also made as a by-product of a process for reducing the lactose content of skim milk that is to be used in the manufacture of ice cream.[87] If approximately 6 per cent of cane sugar is added to skim milk, the mixture may then be condensed to 70 per cent total solids without the development of undue viscosity, and the lactose which crystallizes may be readily removed by filtering or centrifuging. About 65 per cent of the lactose of the skim milk can be removed by this method, but the crystals are heavily coated with milk protein, which makes recrystallization necessary.

Refining. For refining,[60] the raw sugar is dissolved in water at 50° until the solution registers 13° to 15° Bé., or contains 24 to 27 per cent sugar. Powdered bone black and hydrochloric acid are added. The syrup is then heated nearly to the boiling point and milk of lime added until a sharp separation of the carbon takes place. The reaction at which this break occurs varies with the carbon used, but is usually between pH 5.4 and pH 5.8. The syrup is pumped through a filter to a reservoir from which it is drawn into a vertical copper pan and evaporated to a density of 40° Bé. It is then run to the crystallizing vat, where it is cooled and stirred during crystallization. The crystals are separated from the mother liquor in a centrifuge and washed thoroughly with cold water. The mother liquors and wash waters are used several times to dissolve crude sugar, and when they become too salty for this purpose are run to the crude syrup tanks.

The refined sugar is spread on trays and dried in a tunnel dryer, or else a rotating air dryer is used. It is ground in an edge-runner mill to pass either a No. 100 or a No. 200 screen and packed usually in 200-pound barrels.

The yield of refined sugar averages about 2.5 per cent of the weight of the whey, or one-half the amount originally present. This low yield may be due to such causes as lactic acid fermentation, hydrolysis, incomplete crystallization and mechanical losses. About 10 per cent is usually lost during refining.

Several other methods of obtaining lactose from whey have been sug-

gested, among which may be mentioned the evaporation of whey to dryness and the extraction of the lactose with water;[26] the evaporation of whey on kieselguhr or other adsorbent material with subsequent leaching of the lactose;[44] and evaporation with moderate heat, extraction of the albumin with a limited amount of water, and finally extraction of the lactose.[11] Because of growing interest in soluble milk albumin, it is possible that existing factory methods for obtaining lactose from whey will be largely modified to include production of albumin.

Manufacture of β-lactose. Since β-lactose is considerably more soluble than α-lactose and since on that account a temporarily greater sweetening effect can be obtained by use of β-lactose, methods have been developed for making this form on a commercial scale.[1, 77] It should be understood, however, that a mixture of α- and β-lactose in the ratio of their solubilities will dissolve initially to a greater extent at a given temperature than either form alone or the equilibrium mixture. Consequently there is no practical advantage as far as initial solubility is concerned in producing a sugar containing more than 80 per cent in the β form.

Sharp's method[77] consists of adding α-lactose to a saturated solution of lactose held at a temperature above 93.5° and removing β-lactose. Since this solution is undersaturated with respect to α-lactose and saturated with respect to β-lactose, α-lactose dissolves and an equivalent quantity of β-lactose crystallizes out. Filtering in a heated centrifuge gives a sugar that is practically entirely in the β form. Since this process involves a recrystallization, the β sugar produced is of a greater degree of purity than the α sugar fed to the solution.

Bell's method[1] consists of drying on an atmospheric drum dryer a preheated solution containing 80 per cent lactose. A drum speed of 5 to 7 R.P.M. and a steam pressure of 65 to 75 pounds have been found to give a sugar containing 90 to 95 per cent β-lactose. The optimum values of these two critical factors will vary with other characteristics of the dryer and should be determined for the particular drying unit used. Too rapid drying gives mixtures approaching the composition of the equilibrium mixture. Since there is no purification involved in this process, there should be used a sugar of a grade equal to that desired in the β-lactose to be produced.

Quality and specifications. The following specifications[9] seem to be reasonable for a "chemically pure" lactose: Sugar of milk of acceptable quality must be a fine, white, dry, odorless powder of not less than 99.7 per cent strength by the polariscope, containing not more than 0.020 per cent nitrogen, not more than 0.020 per cent fat, and yielding not more than 0.050 per cent ash. It must comply with the U. S. P. heavy metals test and in solution must be neutral to litmus paper. A ten per cent solution must be odorless, colorless, and free from mechanical impurities.

For bacteriological use, lactose must satisfy certain additional tests.[90] It should be free from ethanol, by the iodoform test, and contain not more than 0.15 per cent moisture. A ten per cent solution should show not more than a negligible turbidity when tested for sulfates with $BaCl_2$

solution, or for chlorides with $AgNO_3$ solution. A ten per cent solution sterilized for thirty minutes at 120° should show a pH value not greater than 4.0 and should remain acid on cooling. *d*-Glucose should be absent as proven by failure to produce acid with *Bacterium typhosi* B. or to produce gas with yeast. A sterilized solution should show no growth on incubation.

It appears quite unreasonable, however, to specify such a high degree of purity as that outlined above for lactose which is to be used for modified milk or for any purpose which involves its addition to a mixture already complex. The comparatively high price of lactose on the market is largely accounted for by the expense of refining and, in many cases, there is no valid reason why a sugar that has been crystallized only once cannot be used. It seems at least unnecessary to remove very carefully certain substances from lactose that is to be added to milk, which already contains these same "impurities" in considerable amount. It seems reasonable that, for purposes of specification, there should be considered only those items that are known to affect unfavorably the quality of the product of which lactose is to be a component.

Food value. The literature on the relative value of lactose and other sugars in nutrition is in such an unsatisfactory state that few well-founded statements can be made. Instinctively we are led to believe that, because lactose is the only sugar occurring in milk in significant quantity, it must possess unique characteristics which make it peculiarly valuable in nutrition, particularly in infant nutrition. Lactose does differ in some respects from each of the other sugars, but, from the standpoint of nutrition, it is difficult to decide what properties give it real advantages over the other sugars. The facts that it is possible for lactose to pass the ileocaecal valve in a considerable amount unchanged and that it stimulates only certain organisms which may be present in the large intestine are probably of considerable importance in this connection. Evidence is accumulating which indicates that lactose is more effective than other sugars in stimulating growth of young animals and that its ingestion in considerable quantities by adult animals does not have the fattening effect attributed to certain other sugars. The rôles of liver function in this connection and of differing degrees of direct assimilability are still so imperfectly understood that it seems unwise to attempt authoritative conclusions at present. Whatever the underlying truths are, the facts remain that large quantities of lactose are consumed in milk and milk products, that lactose is used in considerable quantities in proprietary infant foods, and that it is prescribed quite generally by physicians for modifying cow's milk for infant feeding.

Therapeutic value. The therapeutic action of lactose is due largely to its effect on the bacterial flora of the large intestine—considered elsewhere—and to a less degree to the laxative and diuretic effects which it exerts when consumed in fairly large quantities. Both the laxative and diuretic effects are probably results of the dehydrating action of lactose.

Other uses. In addition to the uses already mentioned, lactose is used by confectioners in fondants and in certain other types of candies; by manufacturing pharmacists as a sweetener, diluent, and vehicle in the preparation of medicines in tablet form; and by the manufacturers of certain liqueurs on account of the frosty appearance produced by the crystallization of lactose on the inside of the bottles. The total consumption of lactose in this country is far less than the amount that could be produced from our dairy wastes; consequently it is demand rather than potential supply that determines the extent of manufacture of this sugar.

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PART II

THE PHYSICAL CHEMISTRY OF MILK AND MILK PRODUCTS

Chapter VI

The Acid-Base and Oxidation-Reduction Equilibria of Milk

Acid-Base Equilibria

A knowledge of the elementary principles of equilibria among systems of acids, bases, ampholytes and their salts has become so essential in modern research that we may assume this knowledge to be a possession of every well-trained experimenter in the field of the chemistry and bacteriology of milk. Perlzweig's translation of Michaelis' "Hydrogen-Ion Concentration" [25] deals in detail with the theoretical principles, while Clark's "The Determination of Hydrogen Ions" [10] is concerned chiefly with methods of measurement. This chapter will sketch in brief review the applications to milk.

Since the appearance of Van Dam's papers [40] in 1908, there has accumulated a large number of studies of milk and milk products based upon the fundamental principles of acid-base equilibria. In many of these are recorded much valuable data which it might be considered a function of this chapter to review in detail. However, if the attempt be made to bring out the real significance of these data, two matters of very considerable importance to the milk industry appear. In the first place, a detailed review of certain of the data would require not only a very long critical examination of the assumptions which are tacitly allowed to enter many of the papers, but also an effort to converge those manifold concepts and experimental methods of approach which should be considered in dealing with so complicated a substance as milk. In other words, no aspect of milk chemistry may profitably be considered in isolation from the other advances in chemistry. In the second place, many of the data which might be considered in review will have to remain as invaluable but isolated facts until advances reveal their places in a significant scheme.

To develop a clear picture of acid-base equilibria in milk in the space available, we must choose between a perfunctory review and one which outlines the direction which the growing literature will take.

Hydrogen-ion concentration of milk. The recorded values for the pH of cow's milk differ considerably. The ranges given in a few of many instances are as follows:

6.5-6.8, Van Dam [40]	6.6, Davidsohn [13]
6.4-6.9, Allemann [1]	6.6, Clark [5]
6.8, Taylor [39]	6.5-6.6, Baker and Breed [2]

6.4-6.6, Lisk[28]
6.54, Duncombe[14]
6.7-6.8, Schultz, Marx and Bea-
6.6-6.8, Milroy[26]
6.5-7.2, Van Slyke and Baker[43] (normal 6.5-6.75)

These values depend in part on the extent to which CO_2 has been lost either by exposure of the milk to the lower partial pressure of CO_2 in the atmosphere or by displacement by hydrogen in a hydrogen electrode vessel, in part upon whether or not the milk is perfectly fresh, in part upon whether or not the udder is diseased, in part upon variation in the proportions of the milk constituents, and in part upon either errors of measurements with electrodes and with indicators or inevitable discrepancies between them. Tentatively we shall regard pH 6.6 as a reasonable value for the purposes of the discussion to follow.

Human milk tends toward a higher value:—the pH range being 6.6 to 7.2 and the average 6.97 as reported by Davidsohn;[13] the range 7.0 to 7.6, average 7.22 as reported by Clark;[5] and the range 6.9 to 7.1 according to Ohi.[27] According to Schultz and Chandler,[32] goat's milk ranges from pH 6.4 to pH 6.7; and according to Takata,[38] the pH of the milk of the whale is 6.67.

Buffer action and buffer constituents of milk. Since all milks exhibit considerable buffer action in the neighborhood of their "normal" values of pH, it is a matter of great importance to various phases of milk technology to know the part played by each of the milk constituents. We shall deal with cow's milk chiefly.

Among the innumerable analyses of milk, none seems to be sufficiently detailed and at the same time well enough interpreted for our purpose.

Porcher and Chevallier[30] have given what is probably the most elaborate and detailed treatment of the composition of milk. They note such analytical difficulties as the loss of sodium chloride on ignition of ash, organic origins for part of the SO_4 and PO_4 appearing in the ash, uncertainties in the estimation of citric acid, etc. Since their treatment is elaborate, it might seem desirable to profit from their conclusions regarding representative values for the several constituents. However, these authors have been unfortunate in the terms they have chosen to employ. Since they consider such matters as the electrical conductivity of milk and the lowering of the freezing point, they recognize that many of the milk salts are largely ionized and do not exist as the specific salts they have chosen arbitrarily for a basis of discussion. The use of specific salts makes their discussion needlessly complicated and their argument such that, if there be detected a reason to doubt the value for the total of one element or radical, the whole of the balance is placed under suspicion until the elaborate argument is retraced in every detail. Tentatively there seems to be good reason to suspect the value of Porcher and Chevallier's total inorganic phosphate. They recognize that nearly 20 per cent of the phosphate appearing in the ash may originate in the casein phosphorus, but they have not reviewed the literature on other organic phosphorus

compounds of milk with sufficient critical discussion to carry conviction. Forbes and Keith [15] cite tables in which it appears that the organic phosphorus compounds including casein contribute over 50 per cent of the phosphate found in the ash. It is possible to dialyze out at a reaction of pH 6.6 approximately 50 per cent of the phosphates of milk.

In regard to the variability of the composition of milk, it need only be said here that even the quarter of the udder or the stage of the milking must be considered. Even so, it might be supposed that the analytical results for herd milk would be fairly consistent. They are, so far as orders of magnitude are concerned, but not in details important to the present purpose.

Therefore if progress is to be made in examination of the main features of acid-base equilibria in milk, it is necessary to avoid entanglement in a profitless discussion of unresolved analytical data and, with a realization of the tentative nature of the conclusions to be drawn, to set up an arbitrary, but reasonable, composition as a basis for discussion. This has been done in the first two columns of Table LX.[9] It is not necessary to discuss the origin of the values since they are to be considered as an arbitrary basis for discussion.

Table LX.—Tentative distribution of chief milk components.

	Grams per litre	Equivalents per litre	Mols per litre	Mols per litre in homogeneous solution	Equivalents "Base bound" per litre if homogeneous	
K_2O	1.80	0.0382				
Na_2O ...	0.72	0.0232				
CaO	1.78	0.0635				
MgO ...	0.30	0.0148				
Total ...		0.1397				
P_2O_5	1.50		0.0211 (H_3PO_4)	as $H_2\bar{P}O_4$ 0.0135	0.0135	
				as $H\bar{P}\bar{O}_4$ 0.0076	0.0152	as $\overset{\equiv}{PO_4}$ 0.0633
Citric ...	2.00		0.0104	as $\overset{=}{cit}$ 0.0008	0.0016	
				as $\overset{\equiv}{cit}$ 0.0096	0.0288	as $\overset{\equiv}{cit}$ .0312
Cl	1.00		0.0282		0.0282	.0282
SO_3	0.11		0.0014		0.0028	.0028
Total CO_2			0.0050	as $H\bar{C}O_3$ 0.0034	0.0034	.0034
Casein ..	28.0				0.0084	.0084
Albumin .	7.2				0.0022	.0022
Others ..					.....	.....
Total ...					0.1041	.1395

In the table, minor constituents such as iron and iodine have been omitted because of their low concentrations. It is not so certain that the so-called "excretory" products as a whole may be left out of consideration. Although each is present in a concentration which is low compared with the more obviously important buffers, their low molecular weights

may, combined with effective buffer action, make necessary a reconsideration of their tentative elimination from the discussion.

The fat of whole milk forms a distinct phase,—a phase of sufficient bulk to make necessary a clear distinction between molal concentrations of milk constituents calculated for whole milk and the actual partial molal concentrations in the aqueous phase. For the present the partition of material between fat and aqueous phases may be neglected, although it is of importance in special problems. Regarding acid-base equilibria in a fat phase nothing of useful value is known. It is certain that the data for equilibria in aqueous solution cannot be transferred to fat solutions without radical modification.

The sugar is such a weak acid that it may be regarded as having no significant participation in the buffer effect within the ordinary range through which milk is carried. Its ionization may not be ignored in the study of lactose under heat treatment, nor may it be regarded as a non-electrolyte when the refined treatment of acid-base equilibria is reached.

All the listed metallic elements form hydroxides of such high dissociation constants that at pH 6.6 these elements may be regarded as functioning only in salts.

Of the non-protein anions, chloride and sulfate are ions of such strong acids that at pH 6.6 they may be regarded as "free." Citrate, phosphate and bicarbonate, on the other hand, are recognized as constituents which buffer at pH 6.6. These and the proteins are the chief buffers of milk.

Calculations dealing with the buffer balance of milk. Since values of pK_a $(= \log \frac{1}{K_a})$ specifically applicable to milk seem never to have even been sought, it will be necessary to use the stoichiometric values of Table LXI.

Table LXI.—Dissociation constants of principal buffer radicals of milk.

	pK_1	pK_2	pK_3
Citric acid	3.08	4.39	5.49
Phosphoric acid	1.97	6.85	11.99
Carbonic acid	6.2	10.3	

Inspection shows that the "spread" of the values for phosphoric and carbonic acids is sufficient to make applicable the equation:

$$pH = pK_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

at pH 6.0 and 6.6. Although the same is not true of the citrate system, the equation may be applied to indicate the order of magnitude of the ratios of the several ions. The equation then furnishes the values of Table LXII, which shows which systems are significant.

Table LXII.—Ratios of different species of buffer radicals of milk.

System	Value of ratio at pH 6.0	Value of ratio at pH 6.6	System	Value of ratio at pH 6.0	Value of ratio at pH 6.6
$\frac{[\overset{-}{\text{citrate}}]}{[\text{citric acid}]}$	830.	3300.	$\frac{[\mathrm{H_2\overset{-}{P}O_4}]}{[\mathrm{H_3PO_4}]}$	11000.	43000.
$\frac{[\overset{=}{\text{citrate}}]}{[\overset{-}{\text{citrate}}]}$	41.	160.	$\frac{[\mathrm{H\overset{=}{P}O_4}]}{[\mathrm{H_2\overset{-}{P}O_4}]}$	0.14	0.56
$\frac{[\overset{\equiv}{\text{citrate}}]}{[\overset{=}{\text{citrate}}]}$	3.2	13.	$\frac{[\mathrm{\overset{\equiv}{PO_4}}]}{[\mathrm{H\overset{=}{P}O_4}]}$	0.000 001	0.000 004
$\frac{[\mathrm{H\overset{-}{C}O_3}]}{[\text{free } \mathrm{H_2CO_3}]}$	0.63	2.5	$\frac{[\mathrm{\overset{=}{C}O_3}]}{[\mathrm{H\overset{-}{C}O_3}]}$	0.000 05	0.000 2

The proteins of milk may be considered as functioning only as acids at pH 6.6 and, for purposes of approximation, in the same way at pH 6.0. At pH 6.6 the "base-binding" power of casein may be considered to be 0.0003 mols per gram of casein, a value estimated from the composite data of Cohn's chart.[11] Since there are no independent data for milk albumin and the other proteins of milk, but suggestions that the base-binding capacity of these proteins is of approximately the same order as that of casein, the casein value will be used for the total protein.

Were milk a homogeneous system, we could apply directly the partition of the polyvalent ions as they occur in column 5 of Table LX. Then the sum of the equivalents in column 6 would nowhere nearly match the sum of the base equivalents in column 3. The key to the discrepancy lies in the fact that milk is not homogeneous and that the positions of equilibria calculated for a homogeneous system are seriously upset by the participation of a solid phase. There is a possibility that the low solubility products $\left[\overset{++}{\mathrm{Ca}}\right]^3 \left[\overset{---}{\mathrm{Citrate}}\right]^2$ and $\left[\overset{++}{\mathrm{Ca}}\right]^3 \left[\overset{---}{\mathrm{PO_4}}\right]^2$ cause a preferential precipitation of the trivalent acid components. Were this to occur completely, an almost exact match between total equivalents of acid and total equivalents of base constituents would be found with the assumed data of Table LX, as shown by a comparison of the third and last columns. This comparison is probably extreme.

It should now be evident that a pursuit of the argument in the direction outlined must depend upon a most accurate and carefully interpreted set of analytical data. Preferably this should be based on a single sample of milk. Lacking this, we must turn to another approach.

We shall employ Van Slyke's buffer index,[41] the differential coefficient $\frac{dB}{dpH}$.

It will be significant to assume first that milk is homogeneous and to calculate from the data of Table LX the buffer indices for the citrate,

bicarbonate and phosphate systems which Table LXII indicates should be significant at pH 6.6 and pH 6.0. Table LXIII gives the results of such calculations, the citrate values being estimated from the curve given by Hastings and Van Slyke.[16]

Table LXIII.—Calculated buffer index of milk.

System	$\frac{dB}{dpH}$ at 6.0	$\frac{dB}{dpH}$ at 6.6
Phosphate	0.0053	0.0112
Citrate	0.0049	0.0018
Bicarbonate	0.0027	0.0023
Totals	0.0129	0.0153

Among the titration curves of casein which are to be found in the literature there are discrepancies in the order of magnitude of $\frac{dB}{dpH}$ which are serious for the present purpose. They are doubtless due to the use of different methods in the pretreatment of the casein samples used, perhaps in part to the loss of one of those constituents which Linderstrøm-Lang[22] believes is found in "pure" casein, perhaps to the shifts of dissociation constant involved in the obscure changes which occur to varying degrees when casein is dissolved.[48] Values of $\frac{dB}{dpH}$ for a solution containing 35 grams of casein per liter are to be found which exceed the total buffer index of milk containing this amount of casein at this pH value.

Loeb's[24] curve of composite data and Palmer and Richardson's[20] Figure 2 indicate that if the slope for 3.5 per cent casein solution is proportional to that for 1 per cent casein solution at pH 6.6 $\frac{dB}{dpH} = 0.0136$. While such an assumption is reasonable, it must be recognized as only an assumption, since little is known of ion associations. (Treated theoretically in the fourth chapter of Michaelis and Perlzweig's "Hydrogen-Ion Concentration."[25]) Tentatively using the value given above, we would have the sum of 0.029 for the buffer index of milk at pH 6.6, considering milk as a homogeneous solution. Instead of this, a fairly representative titration of a milk of undetermined composition shown in Figure 7 indicates a $\frac{dB}{dpH}$ value of 0.0186 at this pH value. Some indication of the reason for this discrepancy may be found in the implication to be derived from Whittier's[48] buffer titrations, namely—that the buffer action of casein in milk is quite different from that of purified casein titrated by itself. If we accept his conclusion that the buffer action of casein in milk is negligible at and above pH 6.0, the value for $\frac{dB}{dpH}$ calcu-

lated for pH 6.6 will be slightly too low and the greater difference at pH 6.0 will be quite unaccounted for.

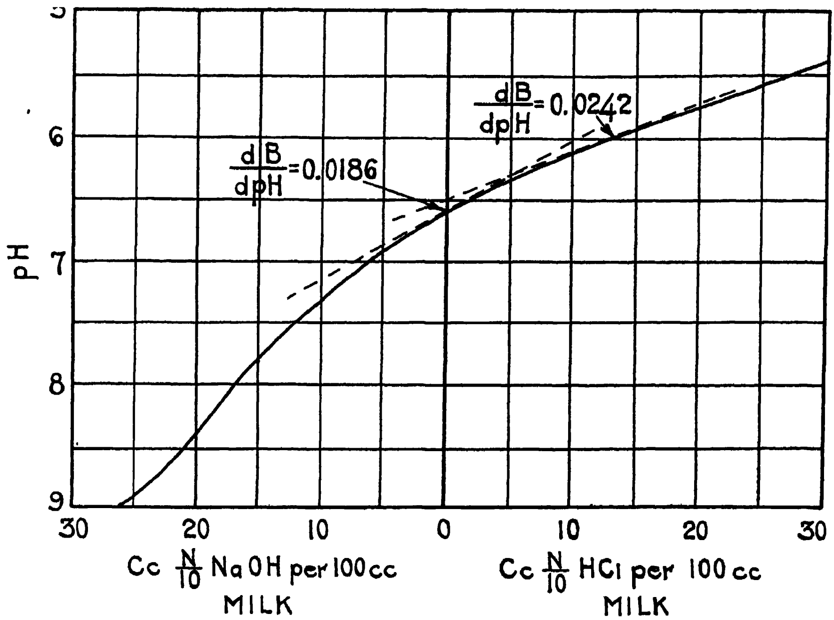

FIG. 7.—Titration curve of milk.

But, after all, such discrepancies are attributable in large measure to the false assumption that milk is a homogeneous system. The high calcium content, together with the extremely low solubility products of certain calcium salts, is capable of establishing a solid phase which alone is nearly sufficient to account for the turbidity of milk and which makes necessary a redistribution of the buffer values of all systems.

To attempt a quantitative redistribution in accordance with this idea would be idle at the present time. The theory of phase buffering has been developed, but its application to milk and similar substances is hampered by a lack of knowledge of the magnitude of the factors which affect so profoundly the values of dissociation and solubility product constants. Consequently only main features can be considered.

The citrate system of milk, considered by itself, has comparatively little buffer effect, partly because of its relatively low concentration and partly because its buffer index never reaches a definite peak, due to the slight differences among its pK_a values. The buffer index of the bicarbonate system is at its maximum at pH 6.2 and, as shown in Table LXIII, is of appreciable, but not very great, importance even at this pH value. Sodium or potassium phosphates show on titration a maximum buffer index at pH 6.8. But calcium phosphate, in a concentration corresponding in respect to each component to that in milk, exhibits a remarkably high buffer index at pH 4.8, the values decreasing rapidly with change in

pH value in either direction.[49] The presence of the other salts of milk, particularly citrates, shifts the maximum toward more alkaline values which approach pH 5.5, approximately the maximum for skim milk. Here we have phase buffering due to precipitation of some form of calcium phosphate and a demonstration of the effect of increasing ionic strength to increase the solubility product constant. The effect of citrate is probably more specific and more complex, however.

The removal of casein from skim milk by acid precipitation gives a whey which exhibits buffering very different from that of skim milk.[18] If the difference between the buffer curves be plotted, we have what may be called a buffer intensity curve for casein "by difference." Such a curve shows a maximum at pH 5.2 and drops to a negligible value at pH 4.3 and pH 5.7. In this region and only in this region does casein apparently exert its buffer action in milk. The curve for the whey is of the same general character as that for the artificial mixture of milk salts mentioned above. Its maximum is at approximately pH 6.0, at which reaction, presumably, calcium phosphate is exerting its maximum phase buffer effect, an effect which in a solution entirely free of ionic strength effects should manifest itself at approximately pH 4.0. In passing, it should be clearly understood that this phase buffering at comparatively low pH values is at the expense of the homogeneous type of buffering which would occur at higher pH values if the phosphate had not come out of solution. From the above facts it appears that the maximum buffering of milk, which occurs at pH 5.5, is chiefly the resultant of the buffering by casein and the phase buffering due to precipitation of calcium phosphate. That this implies that fresh milk is in unstable equilibrium with respect to calcium phosphate does not require justification; it is generally admitted. It is well known that milk under heat treatment undergoes an irreversible shift in acid-base equilibrium involving visible precipitation of calcium phosphate. It would appear that there is concerned in milk a slow adjustment of the calcium-phosphate system, involving the reduction of the supersaturation with respect to tricalcium phosphate and possibly the slow change of solid dicalcium phosphate to solid tricalcium phosphate. These factors have proved troublesome even in the study of simple calcium-phosphate systems, as noted by Holt, La Mer and Chown,[17] as well as in the study of blood and of phosphate fertilizers.

The values for the proportions of calcium in the diffusible and non-diffusible conditions and for the proportion of phosphate in crystalloidal solution are some of the uncertainties that it will not profit us to discuss. Data on these points are conflicting. There is definite evidence that citrate ions form soluble, slightly ionized compounds with calcium ions[35, 36, 51] and there are indications that the calcium caseinate system may be similar in several characteristics to the calcium citrate system. However, the conduct of these systems in relation to each other, to the calcium phosphate system, and to the other systems of milk certainly cannot be definitely and accurately linked to the observable features of milk equi-

libria until the details of the conduct of each component system are established separately on a quantitative basis.

Beginning with the classic papers of Van Dam,[40] there have been numerous and valuable contributions to the elucidation of the relationship of calcium, pH, etc., to specific processes such as the rennet coagulation of milk. Since no exact formulation in the terms prescribed above has yet been provided, the subject of the relation of rennet coagulation to the several factors known to influence it is still a subject of debate. The history of this subject is paralleled by one aspect of blood chemistry. There the tendency is to seek the basic data regarding the state of calcium in blood and to postpone further argument while awaiting the establishment of fundamental data. In the case of milk there are available not even the specific pK_a values for the several buffer systems, nothing on solubility products specifically applicable to milk, and few analytical data sufficient to the purpose.

It should not be overlooked that milk must be "buffered" with respect to Ca^{++} as well as with respect to H^+. Kugelmass[19] has outlined the principles of this subject. It will undoubtedly become of importance coordinate with that of pH-buffer action in several phases of milk chemistry. Were it generally realized that there occur circumstances wherein the addition of calcium salts may not only have no proportional effect on the concentration of calcium ions but may even lower their effective concentration, it would be realized how difficult is a fair appraisal of numerous experiments which are claimed to support a theory for which the time is not ripe.

Titration of milk. In milk $\frac{dB}{dpH}$ decreases very considerably in value as the zone of pH near 8.5 is approached. (See Fig. 7.) In this region phenolphthalein shows appreciable color. Consequently, when milk is titrated with alkali and phenolphthalein is used as indicator, there occurs a resemblance to the conditions existing when phenolphthalein is used as indicator in the titration of a moderately weak acid by a strong base. Partly because of this coincidence and its empirical discovery and partly because there was originally a misinterpretation of the theory of titration, use of phenolphthalein is continued with the original misinterpretations of the true usefulness of titration.

What is measured in the titration of fresh milk is the amount of alkali necessary to shift the protein and salt buffer systems from their initial state of labile equilibrium somewhere near pH 6.6 to that pH value at which phenolphthalein shows the color arbitrarily selected as indicating the hypothetical "end-point." Absolutely nothing else is revealed directly and all other conclusions must be derivative. The value of data obtained when this procedure is carried out under uniform conditions is not altered when, for purposes of imagined convenience, the equivalents of alkali used are transformed into "percentages of lactic acid." This term does not refer to the pedagogical uses of "percentage lactic acid," but the deception remains. In numerous other industries a similar procedure is

followed without the necessity of the deception being felt in the instruction of non-scientific workers.

Inasmuch as the "percentages of acid" found by this method are established in fresh milk by the concentration of the several buffers, and inasmuch as these buffer components have nutritive value, a fresh and normal milk of high titration value is necessarily a milk of high nutritive value. However, a milk suffering from the activity of bacteria, either in the udder or subsequent to withdrawal, will have undergone changes, that usually, but not always, result in an increase in the titration value. Since such changes are undesirable both in market milk and in milk destined for various milk products, the titratable acidity has its significance if used discreetly.[34] It should be pointed out, also, that the amount of alkali necessary to change the pH value of milk to approximately 8.5 is not a reliable criterion of its response to treatments which shift pH in the opposite direction.

In arriving at a standard two radically distinct methods of approach may be considered. The first is largely the concern of the physiologist who, in dealing with the physiology of milk secretion, desires, if possible, to establish a "norm" on the basis of which to judge both the normal and the abnormal aspects of secretion. Pending the arrival of definite conclusions in one or another of the manifold aspects which are here concerned, the handler of milk is forced to set up arbitrary standards. It is probably generally recognized that these are of very doubtful value for the judging of individual milks. They seem to be very loosely formulated statistical standards in which vast but rough and ready experience has taken the place of exactitude. Thus in a community in which customs of feeding, breed of cows, methods of handling the milk, etc., establish a herd milk of such properties that, if the data were treated statistically, a norm would be found, this norm might provide a statistical margin of safety. This being established on a basis which experience proves to be reasonable, if not rational, there is no reason why production cannot be adapted either rationally or by a process of "natural selection" to meet the requirements. The arbitrary norm will then have definite commercial significance, but the advantages involved may be balanced by wastage of milks of high nutritive value. So far as we know, there has been no adequate survey of this situation, most of the discussion having centered about trivial aspects of the problem.

Because of the lower salt concentration of human milk, less alkali is required to shift the equilibria to their state at pH 8.5. This was at one time interpreted to mean that the "acidity" of human milk is less than that of cow's milk; hence the former practice of adding to modified cow's milk, lime, magnesia or sodium carbonate. Sometimes the excuse for the addition of lime-water was to raise the calcium content; but no regard was given to two matters of supreme importance in infant feeding. It has been estimated that 90 per cent of our people show, histologically, evidences of rickets. Among the preventives of this condition are the proper balance of calcium and phosphates in the food and the provision

of the anti-rachitic vitamins. Clark's data [8] on alkalinized modified milks make it certain that the alkalinity in some cases must have been sufficient to destroy vitamins during pasteurization. Furthermore the addition of too much calcium would undoubtedly more or less completely precipitate the phosphate and render it less assimilable under the weakly acidifying conditions of the infant's digestive tract.

As milks enter the stomach they are acidified; a greater or lesser amount of hydrochloric acid, according to the buffer value of the milk, being required to reach a given pH level. The pH value of the infant's stomach normally is near 5.0, and of the adult's 1.2, these very different

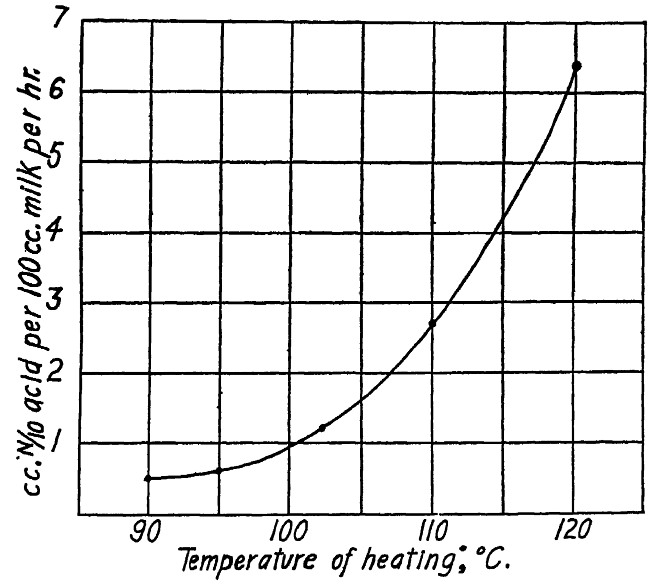

FIG. 8.—Relationship between rate of acid formation in milk and temperature of heating.

values being adaptations to the different enzymes functioning in the two cases. Here we encounter an example of a most important relationship —that of pH to the activities of various enzymes. It becomes a peculiarly unique problem in the rennet coagulation of milk, which was briefly mentioned previously. It is a very extensive subject when the various enzymes active in cheeses and in other products of milk involving fermentation are considered. The vast literature of the subject is treated in various reviews.

Of rather general interest is the fact that products formed by the action of enzymes in acid media are, with notable exceptions, relatively innocuous as compared with the products formed in alkaline solution.

Effects of heat on the acid-base equilibria of milk. Exact and detailed interpretations of the effects of heating milk on its acid-base equilibria must await the clearing up of the general situation already dis-

cussed. However a number of pertinent observations have been made and will be mentioned at this point.

The literature on changes in hydrogen-ion concentration and in titratable acidity caused by heating milk has been reviewed by Whittier and Benton.[45, 46] They have verified previous data and extended the investigations to higher temperatures and longer times than those used by previous workers.

Heating causes the titratable acidity of milk to decrease at first due to separation of carbon dioxide and then increase at a rate depending on temperature till coagulation occurs. (See Fig. 8.[47]) At this juncture the titration values of curd and whey become unequal due, presumably, to the adsorptive activity of the casein. Hydrogen-ion concentration increases smoothly at a time rate dependent on temperature of heating, except that its change is briefly arrested during coagulation. Orla-Jensen and Plattner [28] explain the gross increase in acidity as due to acid formed principally from casein and to a slight degree from lactose. Whittier and Benton [46] maintain from their experiments that the principal source of the acid is the lactose.

Other features of the effects of heat on milk equilibria are a partial precipitation of buffer salts, changes in the characteristics of the protein and partial caramelization of the lactose, all or only some of which may be the causes of the observed shifts in the values of $\frac{dB}{dpH}$. One other frequently observed phenomenon is the slight reversal of the pH change that occurs in a previously heated milk on standing at room temperature for some time, this indicating that the true milk equilibrium differs with temperature.

Acidity in bacteriological investigations on milk. In the bacteriological examination and in the control of bacterial processes of milk and milk products certain elementary principles of pH measurement and control are essential.

It is a fact of experience that a given species will grow most vigorously in a *specific* medium when that medium has a certain *"titratable acidity."* However the value found empirically cannot be used indiscriminately for media of other compositions. On the other hand it is again a fact of experience that the *pH value* of the medium that is favorable to growth will apply rather *generally* to other media. Current practice and special pH values favorable for specific organisms or specific processes induced by these organisms are described *in extenso* in the current literature. Growth of bacteria is frequently completely inhibited by definite hydrogen-ion concentrations though in some cases the constancy of the inhibiting value is partially a resultant of other factors, the actual inhibiting factor being the concentration of the undissociated form of the organic acid produced in the metabolic process.[31]

Methods of measurement of pH values. The pioneer work on hydrogen-ion concentration measurement was carried out with the hydrogen electrode and this electrode is still widely used. In order to avoid

certain difficulties inherent in this method, such as those of obtaining measurements on highly viscous or semi-solid materials, the reduction of certain component substances by hydrogen, and the removal of dissolved gases by the hydrogen stream, other means of determination have been used to a considerable extent. The most important of these are the quinhydrone electrode, the antimony electrode, the glass electrode and the colorimetric methods. For details, consult "The Determination of Hydrogen Ions" by Clark [10] and the current literature.

Limitations in the application of fundamental equations. Having sketched the more important aspects of acid-base equilibria in milk, we may consider briefly some important limitations to the application of the classical equations which are ordinarily employed. The hydrogen electrode, which has furnished the basic data for standard buffer solutions and, indirectly, the more readily applicable values for the dissociation constants of acids, bases, ampholytes, indicators, etc., determines not relative concentrations but effective pressures of the hydrogen ions. These effective pressures are influenced by the other materials in solution and especially by the ions of high charge and effective ionic radius. This would seem to introduce an insuperable difficulty in the rational consideration of products of high concentration, notably cheese and condensed milk. To a degree it does, but there remains a very hopeful aspect. If any equilibrium equation be examined in its fundamental derivation, there is found good reason for believing that it is truly descriptive for limited ranges of concentration of components and for limited ranges of environment of the system in question. This means that if the dissociation "constant" be determined for the conditions imposed and the classical equation involving this constant be used for some limited range of concentration, the results will be of legitimate validity. An enormous amount of work remains to be done with the help of the classical equations, and this cannot be done by the indiscriminate carrying over of data applicable only to dilute solutions. The entire subject of the acid-base equilibria of milk, with the exception of a few definite achievements that still await their places of significance and a few empirical relations of immediate practical usefulness, still lacks that systematic development which has now been going on for a long time in the study of blood. Without such system the empiricist stands in danger of drawing conclusions of no general significance and of advising changes of practice which may entail enormous economic losses. Beyond the rational application of the classical equations lies the application of the Debye-Hückel theory of solutions. Much is already being done with this, for which the current literature may be consulted.

Oxidation-Reduction Equilibria

In a series of articles published in the United States Public Health Reports and reviewed by Clark,[6] studies on reversible oxidation-reduction in such systems as methylene blue-leuco methylene blue are reported. Since the results have a bearing upon the methylene blue tests of milk

and upon several other problems of milk chemistry and milk bacteriology, brief mention is given to this development.

Potentiometric method of measurement. The method employed was the potentiometric measurement of the difference of potential between a gold or platinum electrode and solutions containing definite ratios of methylene blue and its reductant in buffers of known pH value, or of other similar mixtures of a dye and its reductant. Since acidic or basic groups appear in either the oxidant or the reductant or both and since the measurement of the free energy changes of reduction under such circumstances involves the energy of ionization of the several acidic or basic groups at different dilutions of the hydrion concentration, control of the pH values of the solutions was found to be essential. To put the matter another way, it may be said that the measurements furnish definite values for the intensity factor in the energy required to reduce the dye and that the intensity varies with pH.

Consequently, when a reducing system such as a bacterial culture acts upon methylene blue it must develop a definite reduction intensity, measurable in volts, before appreciable reduction of the methylene blue can occur and this potential or intensity varies in an orderly way with the pH value of the medium.

Figure 9 taken from the article by Clark, Cohen and Gibbs [7] shows the variation in the potential, E_h, with variation in pH for several systems, including that of methylene blue, at fifty per cent reduction. The potentials at any other percentage reduction can be found from the relation

$$E_h = E_o' - 0.03 \log \frac{\text{Concentration of total reductant}}{\text{Concentration of total oxidant}}$$

in which E_o' is the value of E_h at fifty per cent reduction and the pH value in question. Such values of E_o' may be read from the chart.

Application to milk. In the article cited above it was shown that the potential differences at gold electrodes immersed in milks containing actively growing bacteria varied with time in an orderly manner affected somewhat by the changes in pH of the milk, and that when that potential was reached at which methylene blue should be reduced, were it present, similar samples of milk did reduce methylene blue. In some cases this type of experiment was performed with milks having different inocula of known cultures of bacteria, in other cases with milks of different degrees of contamination, and in still other cases with milks treated as for the Schardinger reaction. It appears that the E_h of fresh, unheated milk is of the order of 0.20 to 0.30 volts, referred to the normal hydrogen electrode. This value is affected by sterilization methods involving heat. A milk soured by the action of the ubiquitous *S. lactis* has an E_h of the order of —0.20. The change of potential from the positive value of 0.25 volts to the negative value of —0.20 is not gradual, however. The potential ordinarily remains fairly constant for a considerable time and then, in a period of half an hour, changes through the whole intermediate range. This explains why the change of color in the methylene blue test

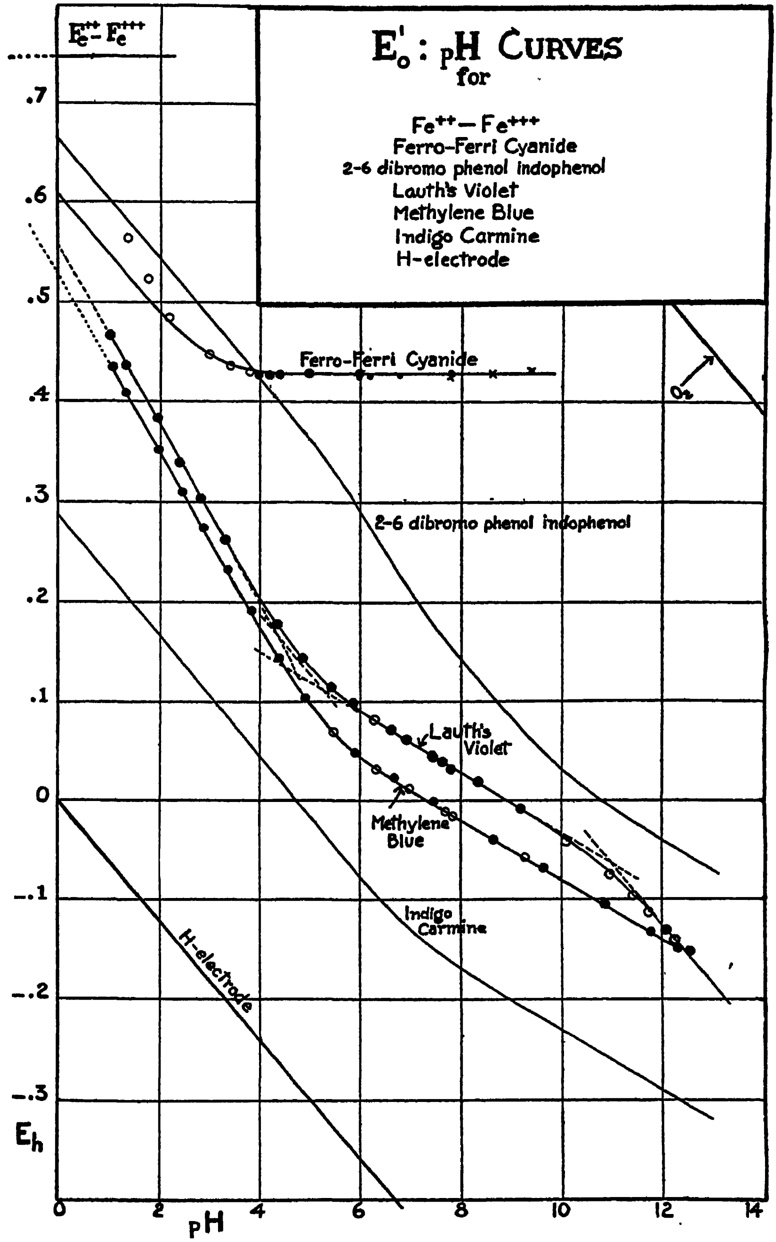

FIG. 9.—Variation of reduction potentials with hydrogen-ion concentration.

is so abrupt and why there is so little time difference between the methylene blue test and a similar test using 2-6-dibromo phenol indophenol.

Potentiometric studies on the lyochromes [87] have shown that the three systems investigated,—hepatoflavin, maltoflavin and uroflavin,—have normal potentials of —0.219, —0.216 and —0.217 volts, respectively, at pH 7; and that one electron is involved in the oxidation-reduction reaction. Presumably, the lactoflavin system will be found to give practically the same values. The physiological significance of the highly negative position of these widely distributed systems is not evident as yet, but it is probably more than a coincidence that E_0' for the lactate-pyruvate-enzyme system is —0.200 volts at pH 7 and that this value is also characteristic of the general anaerobic reduction potential of living cells.

Cannan, Cohen and Clark [4] show in considerable detail how electrode potentials, either with or without the presence of indicator, can be used to study the kinetics of various reactions occurring in biological media. It thus appears that a new and powerful method, revealing much more detail than indicator studies alone, is now provided. Results of the application of this method to milk bacteriology will be discussed in Chapter XI.

Oxidation-reduction indicators. In the meantime, the authors mentioned have developed a series of oxidation-reduction indicators to supplement the few already extensively used in a rough empirical manner; and they have made progress in systematizing the fundamental theory involved, preparatory to the numerous biological and chemical applications which are being revealed.

One item of some interest to milk chemistry appears in another paper by Clark, Cohen and Gibbs.[8] It is there shown that the highly colored products formed by the partial oxidation of certain aromatic diamines, and called by the organic chemist meriquinones, are components of oxidation-reduction systems of great complexity and remarkable instability. In spite of this, a quantitative description has been developed and a consideration of this has led to two conclusions of considerable importance. It is concluded in the first place that reagents such as that of the Storch reaction and others have not been designed with a rational regard to the complexities of the equilibria their use entails, and that the production of the color reaction is a matter of so great a complexity that *exact* use is precluded as impracticable. In the second place it is suggested as a plausible hypothesis that the instability of the meriquinone may result in such a displacement of equilibria that color production may result, not from high oxidation potentials of the tested solution but from low oxidation potentials supported by *catalysts which hasten the condensation of the products of oxidation.* Were this hypothesis to be confirmed it would deal a severe blow to the use of the reagents in question as specific tests of the so-called peroxidases.

Mechanism of oxidation-reduction. In the study of the so-called reductases, oxidases, peroxidases and in the formulation of various oxidative and reductive changes in definite chemical systems there have been used very extensively formalistic concepts of which Wieland's [50] is now in vogue. Some of the results cited in the papers mentioned above, while by no means conclusive in their bearing upon the mechanism, do, never-

theless, furnish data which can not easily be reconciled with current concepts. It would appear, for instance, that the process in the reduction of methylene blue is the acquirement of an electron pair followed, or not followed, as the pH value of the solution demands, by the hydrions.

Irreversible reductions. Regarding the so-called irreversible reductions an excellent review has been given by Conant.[12]

The quinhydrone electrode. The principles involved in the variation of electrode potential difference in systems containing an oxidant and its reductant apply to the case of the so-called quinhydrone electrode devised by Biilmann.[8] Since quinhydrone furnishes what may be assumed to be equal concentrations of hydroquinone and quinone, when present in excess as a solid phase, the potential is as if for an equimolecular mixture of oxidant and reductant. The two phenolic hydrogens do not dissociate appreciably until pH values of the distinctly alkaline region are reached. Therefore the equation, the development of which will be found in "The Determination of Hydrogen Ions" by Clark,[10] reduces to

$$E_h = E_o + \frac{RT}{F} \ln (\mathrm{H}^+)$$

The effect of temperature is defined by

$$E_o = 0.7175 - 0.00074t$$

t being the difference between 30° and the temperature of measurement.

Lester,[20] Knudsen,[18] Linderstrøm-Lang and Kodama [21] and Watson [44] have employed the quinhydrone electrode in the study of the hydrogen-ion concentration of dairy products. It is particularly applicable in work on cheese, casein and buttermilk. The "salt" and other errors, references concerning which are given in the reviews cited, must be taken into consideration in the use of this electrode. It should be borne in mind that the quinhydrone electrode may not be used for reliable pH determinations in alkaline regions for reasons stated above; normally, of course, milk and milk products do not have pH values above 7.0.

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Chapter VII

Physical Equilibria of Milk

Introduction

Milk is a colloidal system composed of several phases varying in their degree of dispersion. The continuous liquid phase may be viewed as a water solution of lactose, various salts and soluble proteins (albumin, globulins, etc.) ; and the suspended phases as fat, calcium caseinate, and some colloidal calcium phosphate, suspended in the homogeneous phase in very fine states of division.

The continuous phase has been referred to as homogeneous. Although the lactalbumin and globulins are soluble in the ordinary sense, in that they form a clear solution in the presence of the lactose and salts, their dispersion is not strictly homogeneous with that of the salts. Albumin and globulin molecules are of amicroscopic sizes but will not pass through animal membranes and are, therefore, relatively large when compared with those of the crystalloids which are readily diffusible. They represent, therefore, a gradation in the sizes of dispersion between those of true crystalloids, and suspensions whose particles are of submicronic sizes. Solutions of these proteins are inherently colloidal in nature. They are solutions of hydrophyllic colloids or hydrosols.

The suspended protein phase of milk is composed of approximately 3 per cent calcium caseinate in the form of innumerable particles varying in size from molecular dimensions to 200 mμ in diameter.

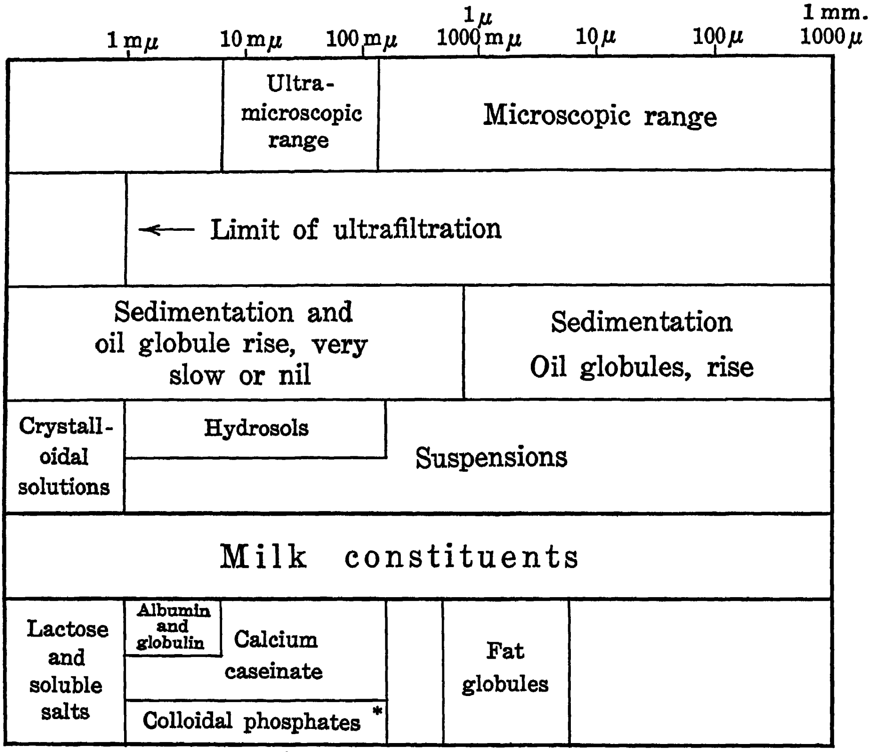
Approximately 3.5 per cent milk fat is also suspended in the liquid medium. This phase is composed of globules of fat of microscopic sizes varying in diameter from 0.10 μ to 22 μ.

Milk consists, therefore, of a number of compounds differing widely in their degree of dispersion. A general idea of their relative sizes and some of the characteristic properties of colloid particles of these sizes may be obtained from the following chart which has been prepared from the results of the work of Zsigmondy, Bechhold, Wiegner, Svedberg and Fåhraeus, and Nichols et al.

Protein Phases

Lactalbumin and lactoglobulin. The physical and chemical properties of these proteins have been treated in Chapter II of Part I. These proteins in their dispersed state form clear solutions which belong to the class of colloidal solutions called hydrosols. These sols are characterized

Table LXIV.—Dispersion of milk constituents.



*Range of size not known

by their relatively high degree of stability to the action of various chemical agents, due to the high degrees of dispersion and hydration of the colloids. This latter property is especially significant and well illustrated by the fact that these sols may be adjusted to H-ion concentrations within those of the isoelectric zones of the proteins without causing their precipitation. Within this zone the ratio of neutral to the total number of particles is at a maximum, viscosity is at a minimum, and the stability is due almost entirely to the degree of hydration. Coagulating and "salting out" of these proteins within this range is, therefore, more complete than under conditions which cause slight ionization. Okuda and Zoller [62] found that the optimum hydrogen-ion concentration for the heat coagulation of proteins in whey is at a pH of approximately 4.5 (electrometric). With addition of small amounts of neutral salts there seems to be a slight increase in the ionization of hydrosols, with a diminution of particle size. The zone for optimum precipitation is shifted slightly and the coagulation temperature is raised. Adjusting the hydrogen-ion concentration of the hydrosols to reactions without the isoelectric range increases their dissociation causing an increase in the viscosity through ionic hydration. In general, the more closely a protein approaches the conditions of

a dissolved crystalloid the more stable it becomes to the action of various agents.

Addition of large amounts of neutral salts "salt out" these proteins. Under conditions wherein the proteins are ionized, greater salt concentrations are necessary to obtain their separation. Different salts act in various manners and no definite rule can be applied to determine the exact results that may be expected. In general, however, the results show a regularity in their action which follows closely the order of the lyotropic series. The reactions of this system with respect to hydrogen-ion concentrations, as well as the effect of the ion to which the ion under consideration is linked, must be considered. The action of salts which dissociate hydrolytically is different from that of neutral salts or salts of the alkaline earths, while salts of heavy metals precipitate the proteins through formation of insoluble precipitates. The action of the salts of the alkaline earths (Ca, Ba, and Sr) is of particular interest.

Electrolyte-free albumin sols do not coagulate when heated. The protein denaturizes, however, or, as it were, dehydrates and in this condition will precipitate when its electric charge is neutralized by the addition of salts. However, denaturization is induced by allowing albumin to remain under the influence of relatively concentrated salt solutions. Ruppel [88] maintains that under this action of salts lactalbumin is slowly converted into lactoglobulin and the change is hastened in more alkaline solutions at 37°. Work by other authors shows also that albumin is modified in its physical characteristics during such treatment.

The work of Sjögren and Svedberg [93] shows that the molecular weight of lactalbumin changes readily. Pure albumin from milk was shown to possess a molecular weight as low as 1000, which in its isolation was built up to molecular weights of 12,000 to 25,000.

The action of alcohol produces dehydration and precipitation and comparatively rapid denaturization.

The degree of dispersion may also be altered by mechanical treatment. Shaking an albumin sol causes a decrease in its osmotic pressure, indicating thereby a decrease in the number of the dispersed units. Prolonged shaking may cause denaturization and precipitation of a large fraction of the proteins. This action undoubtedly occurs at the air/liquid interfaces where the protein is adsorbed. Brouwer [14] found suspended protein bodies in milk, which he maintained were the membranes of gas bubbles, which remained after the gas had dissolved.

Dispersed proteins lower the surface tension of a water medium. This property combined with the hydration and stability characteristics of hydrosols confers upon them the property to stabilize suspensions whose particles are very susceptible to the action of added electrolytes, but which when surrounded by a film of highly hydrated protein are stabilized. The degree to which a sol will protect suspended particles may be measured by ascertaining the gold number or the amount, in milligrams, of colloid which is necessary to prevent 10 cc. of a red gold suspension from coagulating upon addition of 1 cc. of $N/2$ NaCl solution. Lactal-

bumin ranks high as a protective colloid when compared with other substances.

Table LXV.—Gold number of various colloids.

Colloid	Gold number in milligrams
Gelatin	0.005-0.01
Casein	0.01 (as ammonium caseinate)
Lactalbumin	0.02-0.04 (albumin in milk serum) (Author's unpublished data 1927)
Egg albumin	0.06-0.30
Gum arabic	0.15-0.25
Starch (potato)	25.00

The gold number, as well as other physical properties of sols, varies with the conditions under which the sol exists. Conditions increasing the dissociation and hydration capacity increase the viscosity but affect the surface tension but slightly.

Lactalbumin and lactoglobulin in milk vary greatly in their degrees of dispersion. Wiegner [122] estimates that the lactalbumin particles are of sizes varying from 5 $m\mu$ to 15 $m\mu$ in diameter. More direct evidence of their sizes is furnished by Bechhold [9] who studied the ultrafiltrability of various sols. This author lists the sizes of albumin particles relative to those of various gold suspensions. The serum albumin studied consisted of particles of less diameter than those of a gold sol whose particles ranged from 1 to 4 $m\mu$ diameter. The radius of the albumin particle in salt-free egg albumin has been calculated to be 2.47 $m\mu$ and that of crystallized egg albumin containing 3.6 per cent $(NH_4)_2SO_4$, as 1.37 $m\mu$.

The isoelectric point of serum albumin has been determined by Michaelis and Davidsohn [59] as pH 4.70. The isoelectric point of the mixture of the proteins in whey according to Okuda and Zoller [62] is near a pH of 4.50. At the hydrogen-ion concentration of normal milk (approximately 6.60) these proteins must exist in a partially ionized state. There are no data available to indicate the exact chemical state of these proteins in normal milk.

Casein. Casein is a phosphoprotein composed of relatively large molecules. It differs also from those proteins which form hydrosols when dispersed, in that it exhibits a more sharply defined isoelectric zone and a minimum and very low solubility at pH 4.6. It is strongly acid in reaction and unites readily with acids and bases in which it swells and finally dissolves. With the addition of sodium, potassium or ammonium hydroxide to casein their respective caseinates are formed. Robertson [86] and Van Slyke and Bosworth [109] have noted that the amounts of these alkalis necessary to just hold the casein in solution, respectively, is of the order of $1.10 - 1.15 \times 10^{-4}$ equivalents (1.10 — 1.15 cc. N/10 alkali) per gram of casein.

Soluble calcium, strontium and barium caseinates were also prepared by these authors with the use of $2.2 - 2.3 \times 10^{-4}$ equivalents of the hydroxides.

According to Van Slyke and Hart,[107] in a calcium caseinate solution adjusted to neutrality with mineral acids, using litmus as the indicator, the alcoholic precipitate contains 1.5 per cent of CaO and is called the neutral caseinate. If phenolphthalein is used as the indicator (pH approximately 8.5) a "basic caseinate" is formed containing 2.4 per cent CaO. As pointed out by Robertson,[86] these "compounds" at any definite acidity are probably mixtures of salts rather than definite compounds.

The solubility of calcium caseinate is materially affected by the presence of salts. In this respect the Ca ion is especially effective.[86] Besides the other inorganic salts the serum from milk contains about 35 per cent of the calcium in milk in soluble form. It seems probable, therefore, that the calcium caseinate suspension existing in milk is one containing less calcium than one formed with just sufficient $Ca(OH)_2$ to adjust casein to a pH of 6.6, or the approximate pH of normal milk. Van Slyke and Bosworth[111] believe that the amount combined in normal milk is of the order of 8 equivalents and that, therefore, the chemical combination may be expressed as Ca_4 caseinate.

All of the calcium caseinate in milk may be separated from the serum by means of a semi-permeable membrane, this indicating that it exists in a suspended form. The high degree of dispersion is indicated by the fact that the suspension is stable under great centrifugal force.

From ultramicroscopic studies Wiegner[122] concluded that a small fraction of the suspension consisted of particles of submicronic sizes and that the remaining fraction was largely in the form of particles of amicronic sizes. He found that the number of submicrons ranged from 3 to 6×10^{12} per cm.,[3] and that this number was quite constant even in cases wherein the milk had been heated or slight amounts of acid added. The author concluded that coagulation by acids is, therefore, the result of growth of submicrons at the expense of the amicrons. This author considers the probable sizes of calcium caseinate particles in milk to be of 5 mμ to 100 mμ diameter, while Bechhold[9] from ultrafiltration studies concluded that the probable size of casein particles is larger than 40 mμ. Svedberg and Fåhraeus[98] from preliminary ultracentrifugal studies made in 1924 concluded that the particles are of the order of magnitude of 10 to 70 mμ radius (20 to 140 mμ diameter).

Recent studies by Nichols et al[60] of the sizes of calcium caseinate particles in heated and unheated skim milk by means of the ultracentrifuge indicate that the majority of the material is less than 200 mμ diameter with a mean size of about 90 mμ. Preheating of the milk up to 95° had little effect upon the distribution curves of particle size. The values are shown graphically in Figure 10.

An experiment by Clark[20] is instructive with respect to the equilibrium attained in the growth of particle sizes, and their stability. Solutions were prepared from various combinations of salts, alkalis, and acids in such concentrations that, when mixed, their ionic strengths would be somewhat comparable to those in milk, and in the presence of casein would produce a solution of a pH of approximately 6.6. When the solu-

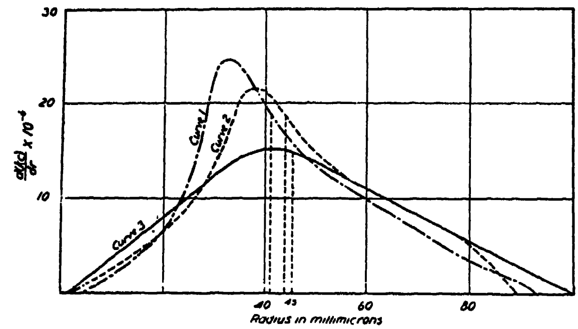


FIG. 10.—"Weight optical" distribution curves of calcium caseinate from milk. From Nichols et al.

(1) Heated to 95°C.
(2) Heated to 65°C.
(3) Unheated

Curve areas: Curve 1-87%, Curve 2-85%, Curve 3-81%

1 sq. = 20%

tions were mixed an opalescence of bluish tinge appeared immediately indicating a formation of exceedingly small particles. Upon standing the bluish tinge turned to white, indicating growth of particle size which, with prolonged standing, showed little change.

The stability of the calcium caseinate suspension, as well as the constancy of the number of submicrons noted by Wiegner in various milks, indicates that the growth of particles attains an equilibrium.[119]

"Mucins." Early investigators noted that fat globules did not readily coalesce and that the fat content of milk could not readily be extracted; which led them to believe that each fat globule was surrounded by a mucin-like membrane. The problem of the composition of such a membrane has been the subject of numerous investigations, practically all of which have been concerned with the nature of the protein contained therein.

Danilewsky and Radenhausen [27] were of the belief that in addition to casein, other proteins were present which resembled those of the blood stroma. Storch washed fat globules free from extraneous protein material and isolated the material of the adsorbed membrane. The substance obtained was insoluble in water and acids and formed a slimy product with the addition of alkalis. It gave many reactions characteristic of albumins and upon hydrolysis produced compounds which reduced Fehling's solution. Storch [96] concluded that the substance isolated was of a glycoprotein nature, differing in many of its properties from those of albumin, globulins, or casein. The reducing power of the constituents of the globule membranes was not observed by Rosengren [87] who followed Storch's procedure in their isolation. Völtz [112] and Abderhalden and Völtz [118] concluded that casein and other proteins of the nature of globulins were constituents of the material isolated. In addition to a mixture of proteins, mucous and fatty substances including calcium soaps are constituents of the globule membrane according to Bredenburg.[68] Hatori [40]

concludes that the "haptein" membrane is composed of a new protein differing in composition from any of the other known milk proteins.

Dornic and Daire [29] early advanced the hypothesis that lecithin forms a part of Storch's "Slimmenmembran," but they offered no evidence to support it other than the fact that cream and buttermilk are richer in lecithin than the original whole milk.

Palmer and Samuelson [68] have shown that the emulsion-stabilizing substances adhering to the fat globules in cow's milk apparently consist of a single globulin-like protein, free from phosphorus, and a mixture of phospholipids. The phospholipids comprised by far the greater part of the total raw material isolated. No glycoprotein was found.

Titus, Sommer and Hart [100] concluded from data on the sulfur, phosphorus and tryptophane content that the protein in the globule membranes was closely related to if not identical with casein. The precipitation test also indicated its similarity to casein.

Palmer and Wiese [69, 70, 71] have studied the composition of isolated membrane material further and conclude that it is a mixture of proteins and phospholipids. The phospholipids isolated were a mixture of mono- and diamino- compounds. The protein material did not correspond with those of any other milk protein in physical properties or in the distribution of its nitrogen among its constituents. Furthermore serological tests with this protein indicated that it possessed specific biological properties.

Colloidal Phosphates

Söldner [94] and other early investigators were of the opinion that normal milk contains suspended tricalcium phosphate. Van Slyke and Bosworth [109] concluded that no tricalcium phosphate is present, but that a relatively large proportion of the dicalcium compound is present in a finely divided condition. This view has been accepted by Palmer,[65] who showed that the fixation of $Ca_3(PO_4)_2$ upon heating of milk is compatible with this idea.

The existence of the postulated calcium phosphates in milk has not yet been experimentally demonstrated, however. Whittier [118] has made a calculation which indicates that the equilibrium concentrations of calcium ions and di-phosphate ions in milk are not sufficient to saturate it with dicalcium phosphate. If only calcium and phosphate ions were involved in the salt equilibria in normal fresh milk, tricalcium phosphate should exist to a considerable extent as a solid or a colloidal phase. It is possible that application of heat results in changes in the other participants, which in turn cause the solubility product of tricalcium phosphate to be exceeded. If the serum obtained from milk by ultrafiltration is merely allowed to stand at room temperature, calcium phosphate is slowly precipitated, this indicating either an original condition of supersaturation or a condition of supersaturation produced by a gradual shift in equilibria in which calcium ions are furnished continuously by one or several of the various calcium-containing components of the system. In ultra-filtered serum

equilibrium changes could obviously take place more rapidly than in milk which contains protective colloids in much greater concentration.

The distribution of the phosphates between the crystalloidal and colloidal phases is still an unsettled question.

Fat Emulsion

This system will be treated in detail later. It will, therefore, suffice here merely to point out the degree of dispersion of the milk fat. In normal milk the globules are of sizes ranging from 0.10 μ to 10 μ in diameter, with an average diameter of approximately 3 μ. The number of globules varies greatly with many factors, but for normal milks may be considered as approximately 2 to 4 billion per cc. Assuming an average of 3,000,000,000 globules per cc., the volume occupied by the fat would be 0.042 cc. or approximately 4.2 per cent of the total volume; and the surface area of this fat phase would be approximately 84 $cm.^2$

General Considerations

A number of substances, including proteins, possess the property of lowering the surface tension of a water medium. This property of the protein constituents of milk leads to relationships that are important from the standpoint of distribution of the phases and the stability of the system. Gibbs deduced [87] from mathematical consideration of heterogeneous systems the important relationship that any substance which lowers the surface tension of a medium will gather in the surface layer. The converse is also true, and though, as pointed out by Gibbs, the surface tension of a liquid may be increased but slightly, it may be lowered considerably by small amounts of certain substances. Due to a lowering of the surface tension of milk by the protein constituents their concentration will be increased in the surface or at the air/milk interface; an important consideration in the phenomenon of skin formation on milk, and in foaming.

Condensed films of proteins are also formed wherever the continuous and the dispersed medium are not homogeneous, providing there is a depression of the interfacial tension at this point. Liquids immiscible with water (oils) form boundaries or interfaces with it at which the interfacial tension is quite different from that at a water/air interface. Thus water at 18° possesses a surface tension of from 72 to 76 dynes/cm. at a water/air interface, while at a water/olive oil interface the interfacial tension is approximately 32 dynes/cm.

The presence of a third substance capable of lowering the surface tension of water usually produces a marked lowering of the interfacial tension of oil/water. The fatty acids and the proteins are especially effective in this respect and, therefore, concentrate at these interfaces. This phenomenon is called adsorption and the relationships are expressed by Gibbs' equation: $A = -\frac{C}{RT}\cdot\frac{da}{dc}$ wherein A is the amount adsorbed,

C the concentration of the surface tension depressant, RT the gas constant, and da/dc the changes of surface tension with changes of concentration.

The intimate relationship existing between the salts of milk and the calcium caseinate suspension has been indicated in a previous section. The particles of this suspension are negatively charged and undoubtedly possess a certain degree of hydration.

It seems certain, however, that in addition to these factors albumins and globulins are adsorbed to some extent and act to a certain degree as protective colloids to prevent agglutination. The flocculent type of curd obtained upon coagulation of a milk high in albumin content (human milk) is an indication of the protective action of the whey proteins. However, as protective colloids these proteins may also function to some extent in regulating the growth of calcium caseinate particles through a poisoning effect upon their surfaces, thus retarding the rate at which equilibrium is established under chosen conditions.

The ability of proteins to form surface films introduces another relationship of utmost importance. When highly purified water and oil are shaken together and allowed to stand the two liquids will separate and form two respective layers. Two pure immiscible liquids only form stable emulsions of very low concentrations of the dispersed phase. (Approximately 0.20 per cent.) In the presence of proteins as stabilizing or emulsifying agents stable emulsions of high concentration may be formed, the adsorbed protein film preventing the coalescence of the dispersed fat or oil. In the presence of emulsifying agents stable emulsions of high concentration may be formed, the adsorbed film preventing the coalescence of the dispersed fat or oil. In milk the fat globules are protected by a film composed of a variety of the system components. (See p. 160.)

According to Gibbs' equation the substance which lowers the surface tension, or interfacial tension, to the greatest extent is adsorbed to the greatest degree. The application of this principle enabled Ramsden [88] to separate saponin from albumin; and Bechhold and Ziegler [10] to fractionate the albumoses or peptones.

The substance adsorbed upon the various surfaces in milk will, therefore, be composed largely of those compounds which lower the surface tension to the greatest degree in those concentrations in which they are present. There is, however, a limit to the extent to which this dynamic phenomenon occurs. An adsorbed protein layer is at first mobile. In a short time a gradual change occurs and a solid skin is formed consisting of denaturized protein.[28, 58, 88] This gradual change is of importance in consideration of the structure formation in whipped cream as well as the properties of the adsorbed membrane of the fat globules.

With this view in mind the question of the presence in the fat globule membrane of substances of an individual character becomes seriously complicated. The progressive changes that may occur through various methods of handling must always be taken into consideration.

Regarding the amount of protein adsorbed upon the dispersed phases there is no accurate estimate. Attempts have been made to determine the amount adsorbed upon the fat globules by washing them free from extraneous material and subsequently removing the adsorbed films. The error introduced is apparent. A change in the concentration of surface tension depressant in the dispersion medium during washing varies the amount adsorbed, in accordance with Gibbs' equation. Devaux measured the thickness of solid skins formed by albumin and found them to be of from 3 to 7 mμ in thickness. Assuming that the adsorbed film on fat globules was of a thickness of 6 to 7 mμ, Wiegner [121] has calculated that in normal milk 2 per cent of the protein was contained in this film.

The importance of the amount of surface presented by the suspended phase is also clearly illustrated by the work of Wiegner. Homogenization of milk containing fat globules of 2.90 μ average diameter reduced their diameter to 0.27 μ, increased their number 1200 times and their total surface 117 times. This author calculated that in the homogenized form the globules adsorbed 25 per cent of the protein or 12.5 times as much as was adsorbed in the original milk.

To what extent adsorption occurs at the calcium-caseinate/serum interface is not known. It is probable, however, that it is slight since the surface tension reduction at this boundary is undoubtedly very small.

With these relationships in mind it is apparent that, with present knowledge, it is impossible to define the equilibrium states of the various phases under what may be called normal conditions. Knowledge of the various components depends upon study under conditions wherein concentration, temperature, distribution or property changes are introduced. The physical and chemical states of each phase are so intimately related to those of any other that any variation in conditions affecting one component may produce a pronounced change in the equilibrium of the other constituents. Furthermore, the equilibria attained by many of the phases with variations in the conditions to which the system is subjected are practically irreversible, and hence conclusions regarding their original state in milk must be drawn with care.

Physical Properties

Surface tension. See pages 189-191.

Freezing point and osmotic pressure. See pages 251-253.

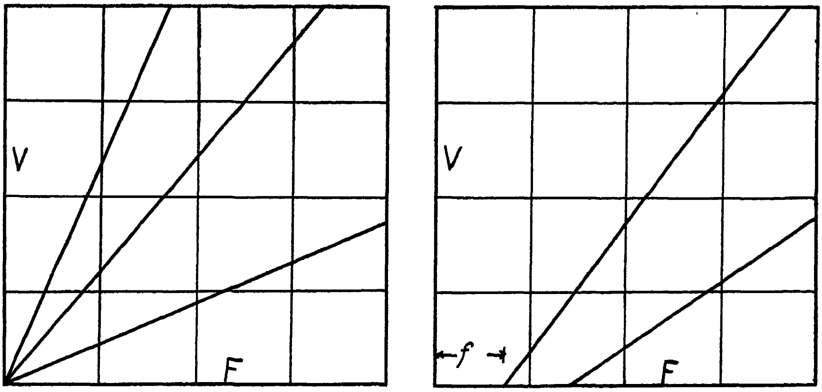
Viscosity. The heterogeneous nature of milk makes it evident that, aside from true viscosity relationships, the colloidally dispersed components of the system introduce effects which, under certain conditions, must be considered from the standpoint of fluidity and plasticity of suspensions. The fluidity (reciprocal of viscosity) of a suspension varies as $\frac{\text{Volume of solid}}{\text{Total volume of suspension}}$. The relationship may be expressed by the formula $\phi = (a - d)\,\phi_1$, wherein a is the volume per cent of the medium

whose fluidity is ϕ_1 and d is the correction for the pore space in particles of irregular shapes. The friction offered by particles as they pass over one another offers another source of loss of energy. If the particles increase in size or number a point will be reached where structure is formed across the channel of flow. At this point viscous flow ceases and plastic flow begins. Viscous flow obeys the law of Poiseuilles, $V = \phi Fr$, wherein V is the velocity given to a surface by a shearing stress F, the surface being at a distance r from another surface considered at rest. The constant ϕ represents the fluidity.

For plastic substances the formula, $V = \mu(F - f)r$, has been proposed, wherein μ is the coefficient of mobility and F the shearing force applied. The yield value depends upon the stress necessary to start the deformation of the substance (the friction to flow, f) and the mobility is the rate of deformation after the yield value has been exceeded.

The nature of the difference between viscous and plastic flow is shown in Figure 11 wherein velocity of flow (V) is plotted against the shearing force (F).

In cases wherein structure is being broken down during flow, a curve of the shape shown in Figure 12 will result.



Viscous flow. Plastic flow.

FIG. 11.—Typical curves illustrating the difference between viscous and plastic flow. From Bingham, *J. Phys. Chem.*, **29**, 1203 (1925).

For a complete discussion of this subject, see "Fluidity and Plasticity" by Bingham.[12]

Relationships are further complicated in milk by the fact that variations in temperature may vary the volume of the suspended phase as well as the degree of dispersion of the fat phase. In addition, with respect to the latter, two relationships must be kept in mind, namely—that below its melting point temperature the properties of a plastic solid are introduced and above this temperature the fluidity of the fat must be considered. Quantitative separation of the values of the various components with respect to these properties have not been made; consequently the data upon this subject must be accepted with reservations.

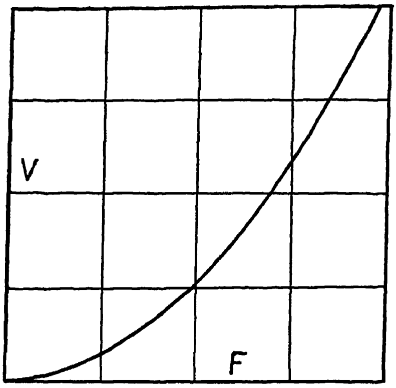


FIG. 12.—Typical curve illustrating the effect of breaking down of structure during viscous flow. From Bingham, *J. Phys. Chem.*, **29**, 1204 (1925).

Many authors [17, 61, 5] agree that casein contributes more to the viscosity value than does any one of the other components. Fat in concentrations found in normal milks contributes less than does the casein but more than does the albumin.

Taylor [99] found that there is the following relationship between solids-not-fat and fat content and the viscosity of the milk:

$$\text{Per cent } SNF = \frac{\text{viscosity} - \text{per cent fat} \times 0.0665}{0.177}$$

He verified the formula upon a number of samples of milk and showed that the values of α and β in Poiseuille's formula for the relationship between viscosity and temperature,

$$N_t = \frac{N_0}{1 + \alpha t + \beta t^2}$$

wherein N_0 and N_t are viscosities at 0° and t° respectively, are for milk, $\alpha = 0.00723$, $\beta = 0.000156$. Milk heated to any temperature below 60° and cooled to 20° showed a decrease in viscosity, while milk heated to 70° and cooled showed an increase. Soxhlet [95] determined the viscosities of milk at various temperatures and compared these values with those of water at the corresponding temperatures. Recalculation of Soxhlet's figures on the basis that the fluidity of water is 1 at 20° indicates that the decrease in fluidity (increased viscosity) with decrease in temperature is not a direct, or straight line relationship.

The fluidity of water increases more rapidly than does that of milk with increases in temperature. This relationship was also noted by Kobler.[40] Kobler likewise stated that the shaking of milk has a marked effect upon its viscosity. Weinlig [115] confirmed the observation of others, that heating milk to 60° to 65° decreases the viscosity of its cooled product, and showed that heating to 80° had the opposite effect. Since aggregation, or increase

Table LXVI.—Effect of temperature on the fluidity of milk.

Temperature °C.	Viscosity of milk	Fluidity of milk	Fluidity (H_2O) (approx.)
0	4.28	0.233	0.558
5	3.52	.284	.658
10	2.80	.357	.768
15	2.41	.415	.877
20	2.12	.473	1.000
25	1.85	.541	1.12
30	1.64	.609	1.25

in the degree of dispersion of phases, varies the fluidity values, the effect of heating and of shaking may be accepted as evidence of changes in the physical states of the colloidally dispersed phases. Babcock [5] has shown that large fat globules in milk are favorable to increased viscosities. Since such globules agglutinate readily, the effect of aggregates may be involved.

In the case of creams, wherein the fat concentration is high, the effect of agglutination is marked. Structures are produced and friction is enhanced, and consequently properties of plasticity become evident.

The effect of increased dispersion of the fat has been studied by Wiegner [121] and by Buglia.[15] These authors found that the fluidity of milk decreased upon homogenization. Wiegner explains the increased viscosity upon the basis of increased volume of the dispersed phase caused by adsorption upon an enormously increased fat surface. Increases in the acidity of cream decrease markedly the viscosity. (See churning.)

Though skim milk is not subject to variations in viscosity due to the fat phase, the value of this property may vary with heat treatment of the system. Evenson and Ferris [81] found that when skim milk was held at low temperatures the viscosity changes were greater than when it was held at higher temperatures. A shift in the equilibrium of the calcium-caseinate system causing separation of larger amounts of the suspended phase may be involved.

Whitaker, Sherman and Sharp [116] noted that there is a gradual decrease in the viscosities of skim milks which have been heated at progressively increasing temperatures up to 55° C. for 30 minutes. Heating at temperatures above 55° reverses this trend and progressively increases the viscosity.

Results, similar in their trend but of less magnitude, were also noted by these authors with whey.

Bateman and Sharp [7] have shown that the viscosities of diluted skim milk samples are not strictly a linear function of the total solids concentration. Similar results were obtained by Leighton and Kurtz,[53] who showed that practically linear relationship exists for fluidity up to a concentration of approximately 10 per cent solids. From 10 to 17 per cent solids the solutions are pseudoplastic and at concentrations of solids greater than 17 per cent the milks exhibit properties of plastic flow. The measurements were made at 0°.

These relationships are shown in Figure 13.

The concentration of solids at which these properties become evident will vary with the treatment to which the milk has previously been subjected and with the temperature at which the measurements are made. Leighton and Kurtz have calculated the fluidities of milks of different solids concentration from the data of Bateman and Sharp obtained at 25°. The values indicate that plastic properties become evident at approximately 22 per cent solids at this temperature.

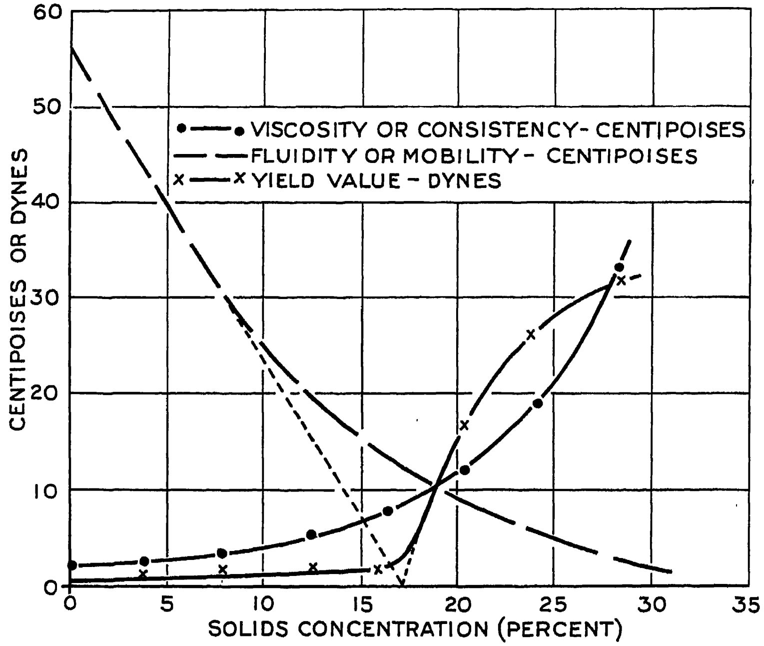


FIG. 13.—The variations in the plastic properties of skimmed milk with varying concentration of solids.

The increase in viscosity of skim milk caused by heating to temperatures above 70° for varying lengths of time is of especial interest. This change may be due either to increased concentration of the suspended phase, through precipitation of colloidal calcium phosphates, or to hydration of the casein; or to both. The latter explanation for increased viscosities is given by Chorower,[18] who believes that the casein particles swell when the calcium is split off in the heating process, thus causing thickening.

This increase of viscosity brought about by heating is also closely related to the baking quality of a skim milk powder when it is added to dough mixes.[38] It is still an open question whether in these cases the action is one of promotion of structure formation through increase of the suspended phase concentration and increase of particle size, or one of increased hydration.

Condensed milks prepared from milks heated to high forewarming temperatures (95°) gel readily in storage, while those prepared from milks heated to lower forewarming temperatures show this tendency to a lesser degree.[54]

Density. The density of milk is dependent upon the amounts of the dissolved and the suspended matter. The amounts of protein, lactose and salt constituents vary over quite narrow ranges; consequently the variations in the densities of skim milks are small. Knowing the density and fat content of a milk, and correcting for temperature difference, one may calculate the solids-not-fat.[6, 42, 64] The results agree quite closely with those obtained by gravimetric analysis.

Approximate figures for the various constituents may also be obtained by calculation.[108, 68]

The greatest variations in the density of constituents occur in the fat, therefore the densities of whole milks and creams vary over wider ranges than do the densities of skim milk. These variations in the density of the fat introduce a factor which is not accounted for in the various formulae used in the calculation of solids-not-fat and account, to a certain degree, for the uncertainties of the results obtained in this manner.

The specific gravities of skim milks may vary from 1.032 to 1.0365 at 15°. Rahn[82] has determined the densities of a number of skim milks at 50° and gives 1.0233 and 1.0232 as the averages for two groups of samples. The variation between extremes was 0.0058, or 0.56 + per cent. The densities of the fats at the same temperature are given as 0.89857 and 0.89729, with maximum variations of results 0.0116, or 1.30 per cent of the density. The specific gravity of milks of ordinary fat contents may vary from 1.028 to 1.034.

Aside from variations due to fat content, the density may vary with temperature changes. These variations of the density of skim milk parallel very closely those of water at various temperatures. The relationships of these values to the specific gravity are given by Whitaker, Sherman and Sharp.[116]

Immediately after the milk is drawn the specific gravity is somewhat higher than after standing for a short time.[77] At first it was thought that changes in protein hydration were concerned. Toyonaga[101] showed, however, that the change was due to volume changes of the fat upon solidification, and that the change did not occur in skim milk. Fleischmann and Wiegner[34] confirmed these results and showed the density increase when milk is held at 15°. Fleischmann[33] has shown also that volume changes with variations of temperature are greater for milk than for water. These changes are attributed principally to variation in hydration of the proteins.

The specific gravity of milk fat varies, according to Koestler,[51] from 0.9355 to 0.9448 at 15°. The values obtained by Rahn at 50° have already been given. The variations noted by these authors explain the variations of the density of whole milks and creams. These variations are of great practical importance when considered from the standpoint of methods for

the determination of fat content wherein volume calibrations are depended upon.

Index of refraction. The power of a solution to refract light is a function of the molecular concentration and is only slightly dependent upon the temperature. Each particular substance in a mixture preserves its own refractivity and hence the refractive index of a mixture is that of the total of the refractive indices of the substances. This property bears, therefore, a definite relationship to density which is expressed in the Lorenz-Lorentz formula,

$$\frac{N^2 - 1}{N^2 + 2} \times \frac{1}{d} = \text{specific refractive index}$$

wherein N equals the refractive index and d equals density. This relationship is independent of temperature and concentration but, in cases where proteins are concerned, will vary with their state of aggregation.

As indicated in the previous section, the percentage of solids-not-fat in a milk is quite constant and since there is a definite relationship between density and refractivity it is possible to calculate the total solids through the use of the determined values for the latter.

The refractive index of milk is given by Jorgensen [48] as ranging from 1.3470 to 1.3515.

Colostrum milk, because of the increased concentrations of the constituents, gives figures of slightly greater value.

The value for the serum is slightly lower and is given as 1.3430 to 1.3443 by Ripper.[85]

Since the refractive index is a function of the molecularly dispersed substances the serum is usually used in its determination. Separation of the suspended phase has been accomplished in various ways, namely, by the acetic acid method,[74] the $CuSO_4$ method, Ackermann's $CaCl_2$ method,[1, 2] Jorgensen's rennet method,[48] or by the development of acid. Though each method yields serums which give results that are quite concordant within themselves the values are not comparable. Of these various methods the $CaCl_2$ method of Ackermann seems to give the most satisfactory results.

Mai and Rothenfusser [56, 55] noted that the values obtained were very nearly constant when especial care was taken to obtain the serums under identical conditions of procedure. Variations of 0.50 per cent were noted between the actual and calculated values. They noted also that variations in the refractive index with dilution were of the order given by Cornalba,[28] namely, 0.10 per cent decrease in refractive index with 1.60 per cent addition of water and a 5.50 per cent decrease with 10 per cent addition of water. The refractive index was independent of the fat content of the milk used.

Wiegner [120,119] found that the specific gravity of the dry constituents of the $CaCl_2$ serum is very nearly constant at 1.6850. Correcting for the volume contraction caused by each constituent, as far as known, this author determined the specific refraction for each constituent as follows:

Lactose	$R_1 = 0.2$
Serum proteins	$R_2 = 0.21480$
Serum ash	$R_3 = 0.1377$
Citric acid	$R_4 = 0.1922$
Water	$R_5 = 0.20606$

Since these values are additive in nature the specific refractive index of a milk serum, of the indicated composition, would be as follows, according to this author:

	Per cent	
Lactose	$5.20 \times R_1 =$	1.076
Protein	$0.30 \times R_2 =$	.064
Ash	$0.55 \times R_3 =$	.076
Citric acid	$0.10 \times R_4 =$	.019
Water	$93.85 \times R_5 =$	19.339
	$100. \times R =$	20.574
	$R =$	0.20574

The specific refractive index of water is approximately equivalent to that of lactose, of serum protein or of citric acid, and therefore the addition of small amounts of water varies the specific refractive index of the serum within very narrow limits.

Wiegner gives the relationship between total solids, and the specific refractive index and the density as follows:

$$t = 245.36 - \frac{N^2 + 2}{N^2 - 1} \cdot 50.405$$

$$t = 245.36 - 244.92 \cdot \frac{1}{d_{4}^{20}}$$

These relationships give values for total solids within ± 0.20 per cent accuracy.[80]

The Lorenz-Lorentz formula has been applied by Schneck [90] to calculate the specific refractive index from the density and refractive indices of a number of samples of milk fat. These values approximate closely the values calculated from the refractivities of the constituent atoms. Assuming a mean specific refractive index, he found that the density of milk fat could be calculated from the refractive index, and vice versa. The results differ from directly determined values no more than 1 to 2 units in the third decimal figure.

Conductivity. The conductivity of a pure solution is a function of the ionic concentration. In addition to dissociation factors, the physical state (fluidity) of the medium is also of importance. Colloidally dispersed substances obstruct ions in their migration and therefore decrease the conductivity. Thus, when fat is separated from a milk the conductivity increases.[99] This increase is probably due to removal of obstacles (fat globules) from the paths of the ions. Taylor found that with removal of the fat (5 per cent) from a milk the conductivity was increased 11.4 per cent. Addition of 5 per cent of water decreased the conductivity

3.60 per cent. Hence the lowered conductivity may be attributed to the obstructing influence of the fat globules.

Taylor [99] and also Coste and Shelborn [21] have shown that there is no apparent relation between electrical conductivity and ash content or total solids-not-fat of a milk. They attribute the greater part of the conductivity to the Cl ions and the latter have calculated that in various milks from 49 to 78 per cent of the conductivity may be due to this component. As milk is diluted with water its specific conductivity decreases, but at a rate less than would be attributable to mere dilution without dissociation.

Because of the variations in the composition of milk it is evident that the conductivity may vary over comparatively wide ranges of values. Koeppe [50] states that these values may vary from 33.9 to 94.3×10^{-4} mhos. Considerably smaller variations have been noted by other workers whose results indicate that milks from healthy animals have an electrical conductivity of 45 to 48×10^{-4} mhos. Milk from pathological animals is usually of relatively high salt content and possesses, therefore, a relatively high conductivity.[78]

The electrical conductivity of cream has been studied by Palmer in relation to churning. (See p. 200.)

The Fat Phase

Size and distribution of fat globules in whole milk. In freshly-drawn milk which has not been subjected to agitation the fat occurs mainly as individual globules varying in size from 0.10 μ to 22.0 μ.[38] Though the range in sizes of globules between extremes is comparatively large, practically all of them are of less than 10 μ diameter and of an average diameter of approximately 3 μ.

Being of microscopic size it is, therefore, not surprising that they were first detected by Van Leewenhoeck,[105] who also devised the first method for discerning bacteria.

The number of fat globules that are present in a unit volume of milk depends upon its fat content as well as upon the sizes of the globules. In general it may be stated that in normal milk of high fat content there is a greater number of the larger globules than in normal milk of low fat content. Furthermore, the distribution of globules with respect to size and number varies with different breeds, individual cows, health of animals, stage of lactation period, and feeds. Therefore, any figures given must be considered with these qualifications in mind.

Figures obtained by various investigators [4b, 103, 89, 125] indicate that the number of globules of all sizes in 1 cm.^{3} of milk ranges from 1500×10^6 to 5000×10^6, with $1500\text{-}3000 \times 10^6$ representing the numbers most frequently obtained. Early investigators established the approximate average range of sizes of the fat globules and noted variations in sizes and numbers under the different conditions already stated, but correlation of numbers, sizes, and distribution awaited the introduction by Babcock of more accurate methods and of a method for the determination of relative size.

To obtain his values this investigator counted the number of globules in a definite quantity of milk and divided the fat content by this number. Though the method did not yield absolute values for sizes, it served well for experiments wherein comparisons only were sought.

Van Dam and Sirks [103] used a direct procedure for determining size and number, which was also used by Rahn. A definite number of globules were counted (usually 600 to 800) and the actual number within each chosen group was noted.

In view of the fact that the volume of a sphere varies as the cube of its diameter, the relative number of globules of various sizes gives an incomplete idea of the distribution of the fat content. Table LXVII taken from the work of Van Dam and Sirks and Table LXVIII taken from the work of Rahn [81] indicate the general nature of the distribution of globules in milk according to size, and the distribution of the fat content between the various groups:

Table LXVII.—The distribution of the fat content in whole milk according to numbers and sizes of globules.

Av. diam. of globules	Relative number in groups	Per cent of total number	Relative amount of fat in each group
μ		per cent	per cent (calc)
.38	51	6.40	.017
1.14	170	21.30	1.58
1.90	267	33.40	11.46
2.66	142	17.70	16.74
3.42	103	12.90	25.79
4.18	40	5.00	18.28
4.94	18	2.20	13.58 +
5.70	5	.60	5.80
6.46	4	.50	6.75
	800	100.00	100.00

Table LXVIII.—The distribution of the fat content in whole milk according to numbers and sizes of globules.

Av. diam. of globules	Relative number in groups (calculated)	Per cent of total number	Relative amount of fat in each group
μ		per cent	per cent
0- 1	47.70	7.95	0.0225
1- 2	149.10	24.85	1.90
2- 3	146.70	24.45	8.65
3- 4	129.00	21.50	20.60
4- 5	72.90	12.15	25.10
5- 6	38.70	6.45	24.45
6- 7	11.10	1.85	11.40
7- 8	3.60	0.60	5.60
8- 9	1.20	0.20	2.30
9-10	0.00	0.00	0.00
	600.00	100.00	100.+

Babcock,[4] Van Slyke,[10b] Woll,[125] Gutzeit,[39] Schellenberger,[80] Hunziker [46] and others have studied the variations of globule size with breeds and have noted especially the relatively large globules in milk from Jersey and Guernsey cows when compared with the globules in milks from other breeds. Figures in Table LXIX indicate the approximate average size of globules for various breeds obtained by several of these investigators.

Table LXIX.—Showing the average size of fat globules in milk from cows of different breeds.

Breed	Average diameter of globules			
	Van Slyke	Woll	Gutzeit	Schellenberger
	Relative size	μ	μ	μ
Jersey	955.8	4.05	3.50	2.95
Guernsey	716.6	3.71	...	...
Holstein	420.1	...	2.58	2.30
Ayrshire	420.9			
Holderness	427.6			
Devon	375.			
Shorthorn	....	3.46	2.76	...
Swiss (Brown)	....	...	...	2.33

The average size of the globules as well as their distribution and the distribution of the fat content for six breeds is well illustrated by the results of Van Slyke [106a] given in Tables LXX and LXXI.

Table LXX.—Average relative size and number of globules during the entire lactation period.

Breed	Average diameters—Micrometer scale divisions; and in μ (calc.)					
	0-1 — 2.4 μ	1-2 2.4-4.8 μ	2-3 4.8-7.2 μ	3-4 7.2-9.6 μ	4-5 9.6-12.0 μ	5-6 12-14.4 μ
Jersey	81	383	321	181	53	11
Guernsey	65	389	350	144	44	7
Devon	107	521	280	79	12	1
Am. Holderness	114	538	282	57	8	1
Ayrshire	146	540	234	62	16	2
Holstein-Fr.	145	546	245	51	11	2

Table LXXI.—Per cent of total fat in groups of globules of various sizes.

Breed	Average diameters.—Micrometer scale divisions and in μ					
	0-1 — 2.4 μ	1-2 2.4-4.8 μ	2-3 4.8-7.2 μ	3-4 7.2-9.6 μ	4-5 9.6-12 μ	5-6 12-14.4 μ
	per cent	per cent	per cent	per cent	per cent	per cent
Jersey	0.1	11.3	26.1	30.7	23.9	7.9
Guernsey	0.1	11.3	33.2	29.7	25.7	
Devon	0.1	23.0	42.5	34.4	...	
Am. Holderness	0.3	24.7	40.1	27.6	7.3	
Ayrshire	0.3	34.0	41.6	17.8	6.3	
Holstein-Fr.	0.3	38.3	50.1	11.3	...	

Van Slyke[106f] also studied the relative size of globules in milk from six different breeds during the advance of the lactation periods and obtained the values of Table LXXII.

Table LXXII.—Relation of month of lactation to relative sizes of fat globules.

Month of lactation	Breeds of dairy cows					
	Jersey 25 cows	Guernsey 20 cows	Holstein 9 cows	Ayrshire 33 cows	Holderness 20 cows	Devon 16 cows
1st	1104	928	...	687	...	546
2nd	1098	1063	640	580	661	585
3rd	1228	954	576	624	607	450
4th	1097	659	256	426	501	547
5th	1149	839	396	384	397	319
6th	846	737	595	399	324	355
7th	1017	584	340	322	329	270
8th	733	568	310	298	379	200
9th	715	408	384	241	315	250
10th	571	426	284	248	336	228
Average for year	955.8	716.6	420.1	420.9	427.6	375

With the progressive decrease in the sizes of globules during advances in the lactation period there is a corresponding increase in their number. For the six breeds studied Van Slyke[106f] gives the figures of Table LXXIII.

Table LXXIII.—Average relative number of globules during each month of the lactation period.

Month of lactation	Jersey	Guernsey	Holstein	Ayrshire	Holderness	Devon
1st	49	61	...	62	...	68
2nd	53	46	53	66	67	68
3rd	45	52	67	70	68	99
4th	86	76	132	85	87	77
5th	56	64	95	93	79	152
6th	76	70	66	94	104	144
7th	62	103	94	117	111	186
8th	80	89	123	140	94	241
9th	83	120	132	168	116	225
10th	103	104	168	162	106	211

The results given in Tables LXXIII and LXXIV show that the fat globules increase in number and decrease in size as the lactation period progresses, except for a short period during the initial months when the reverse relationship exists.

Woll[124] confirmed these results and illustrated the reciprocal nature of the relationship between the number and size of globules during this period as follows:

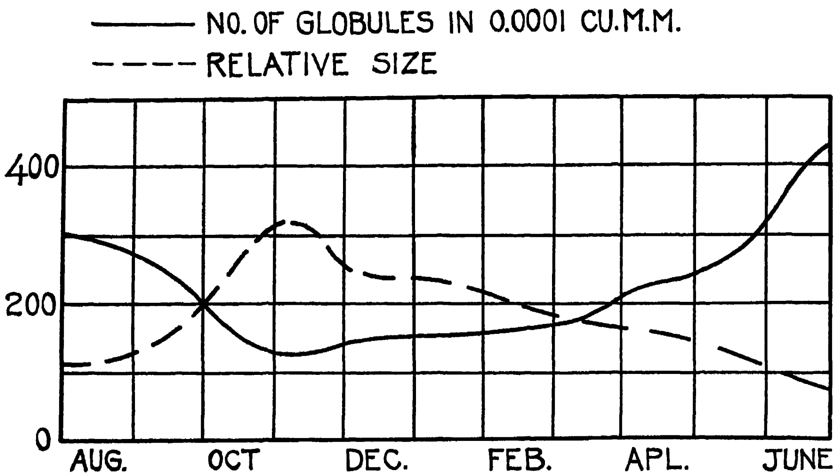


FIG. 14.—The variation in size and numbers of fat globules during the period of lactation. The cow from whose milk these data were obtained calved on Oct. 20 of the year indicated in the chart and on Sept. 16 of the following year. Data from Woll.

The results of Schellenberger [89] given in Table LXXIV illustrate the variation in number of globules of given average diameters with advances in the lactation period.

Table LXXIV.—Variation in numbers of globules of different sizes with advance of lactation period.

Month	Number of globules of different sizes					
	8.82μ	7.60μ	6.00μ	3.67μ	1.96μ	Total
February	20	..	210.3	1529	1081	2480
March 1	20	..	80.8	942	1055	2104
March 31	..	23	126.6	1081	1631	2862
April	..	10	124.0	972	2069	3177
May	..	15	103.0	879	2347	3362
June	..	19	97.0	824	2715	3450
July	..	14	55.1	381	4008	4449

Babcock [4b] pointed out the fact that cream containing large globules churned more readily than did cream containing small globules. Van Slyke [106e] showed that in general the relative ease of churning of creams from milks of the six breeds studied, corresponded with the relative sizes of their globules, and that there was a general tendency to an increase in the length of time required for churning as the periods of lactation advanced. The rapid increase in the number of small globules late in the lactation period is of special interest in relation to ease of churning. The results of Van Slyke [106d] upon creaming efficiency also show that losses in the skim milk with spontaneous creaming increased with advances in the lactation period. This was most pronounced toward the end of the lacta-

tion period especially in milks of breeds producing globules of the smallest relative sizes.

In a more recent study of the number and size of globules in milk Van Dam and Sirks [103] find that the frequency curve for the various globule sizes in milk from fresh cows shows two peaks. As the lactation period progresses these peaks merge into one. Their results obtained upon milks

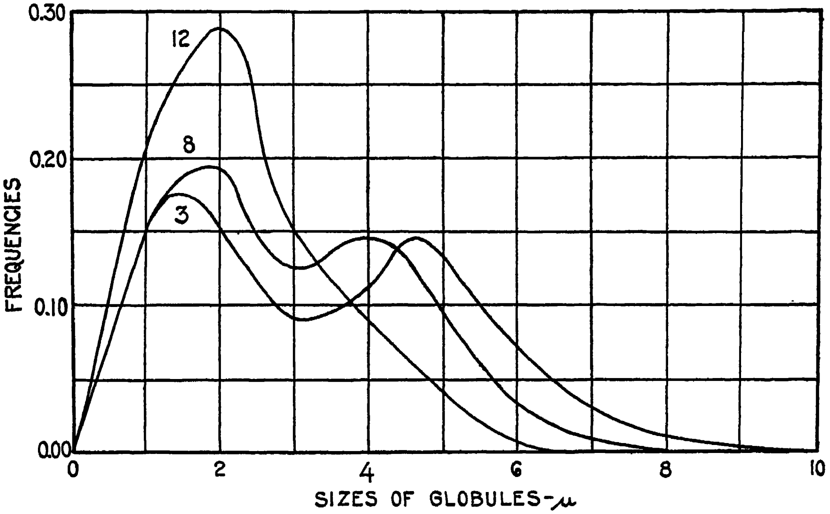


FIG. 15.—Frequency curves for the sizes of fat globules in milk at three stages in the lactation period. Figures on graph denote weeks from calving.

drawn at the third, eighth, and twelfth weeks in the lactation period are expressed graphically in Figure 15.

The type of feed also influences the size and number of the fat globules in the milk. Woll [123, 125] studied the effect of the dry feeds especially, and his results indicate that each feed affects the physical conditions of the fat globules in a specific manner. Gutzeit [39] was unable to confirm Woll's results and in some instances obtained opposite results. Hunziker [46] confirmed the general conclusion of Woll that dry feeds may produce small fat globules in the milk and that succulent feeds favor the formation of larger globules; and noted that pasturing, feeding of silage and of rations high in oil content favor the production of the larger fat globules. He concluded also that changes of feed and other factors affecting the general condition of the animal affect the size and number of fat globules.

The size of fat globules in goat's milk. The average diameter of the fat globules in goat's milk is less than that of the globules in cow's milk, the majority of them being of less than 2 μ diameter. The following table for their distribution is that given by Schultz and Chandler.[91]

Coalescence, aggregation, and subdivision of fat globules. When milk is agitated the fat globules come into contact with one another and

Table LXXV.—Average per cent of fat globules of various sizes in 45 samples of goat's milk.

2μ	2-4μ	4-6μ	6-8μ	8-10μ
57.	34.	7.0	2.0	0

there is a tendency for them to coalesce, or to adhere to one another and form aggregates (clumps). The degree to which these phenomena will occur in a milk or cream is dependent upon the temperature, the acidity, the fat content and its degree of dispersion, the degree of agitation, and the fluidity of the system. The fat content, the degree of agitation, and the fluidity of the system determine the probability of collision of the globules. The temperature affects the phenomena mainly through variation in the conditions at the surfaces of the globules. These conditions are also determined to a certain extent by the changes in the properties of the adsorbed membrane caused by varying degrees of acidity. When various milks or creams are compared specific properties of their serums must also be considered. (See creaming.)

Coalescence, and dispersion, or breaking up of the globules, are necessarily phenomena which may occur when the fat globules are in a liquid or semi-liquid state. Agitation of milk or cream at temperatures above the melting point of the fat promotes the formation of a relatively small number of large globules and may also cause subdivision of globules.[4a]

Aggregation, or clumping, wherein each globule maintains its individual structure though its shape may be somewhat distorted, occurs over a wide range of temperature. Rahn[81] and others have studied the effect of different temperatures upon this phenomenon and find that subdivision is favored by increases in temperature, while clumping is suppressed. Above 63° to 65° there is little or no aggregation of globules. Weinlig[115] maintains that a time as well as a temperature factor is involved and that 75° is the temperature at which the tendency to aggregate ceases, though 63° to 65° for 30 minutes will accomplish the same result. Below 65° clumping increases with lowering of the temperature and at 7° to 8° the clumping is at a maximum. (See churning.)

Though the temperature factor is of prime importance in these phenomena, the degree to which they occur in milk at any chosen temperature is dependent upon the degree of agitation. Agitation of milk at the higher temperatures in forewarmers, pumps and other dairy equipment results in a greater degree of dispersion and hence smaller globules.[4a, 81] At the lower temperatures clumping is promoted. In cream the condition of greater density of globules, or their packing, as it were, promotes coalescence and aggregation to a greater degree than is noted in milks. This product contains, therefore, a relatively large number of large globules ($> 9\ \mu$ diameter). The results obtained by Rahn[81] under conditions of modern industrial practice, which are given in Table LXXVI, illustrates the coalescence of globules which occurs during separation of the cream.

(See churning.) For the sake of comparison the figures obtained upon skim milk are also included here and will be referred to in a later section.

Table LXXVI.—The relative number of fat globules of various sizes and the relative fat distribution in whole milk, skim milk and cream. (From Rahn.)

Globule sizes	Whole milk 3 per cent fat		Skim milk 0.13 per cent fat		Cream 40.5 per cent fat	
Diameter in μ	Relative number of globules	Fat distribution per cent	Relative number of globules	Fat distribution per cent	Relative number of globules	Fat distribution per cent
0-1 }	418	0.0019 }	592	0.0020 }	357	0.0090 }
1-2 }		0.0720 }		0.0530 }		0.4500 }
2-3 }		0.3900 }		0.0420 }		2.0000 }
3-9	182	2.5400	8	0.0330	231	23.5700
Greater than 9	0	0.0000	0	0.0000	12	14.5000
	600	3.0000	600	0.1300	600	40.5200

Homogenization. The homogenization or viscolization process as used in dairy practice consists essentially of forcing milk or cream through a small aperture under high pressure. As the fat globules subdivide and are dispersed throughout the medium, surrounding protein membranes are formed at the fat/liquid interfaces which stabilize the suspended globules and prevent their coalescence. Variations in the temperature of the milk or cream cause variations in the viscosities and surface tensions as well as the interfacial tensions of the phases. The magnitude of these values is usually less at the higher temperatures and since they represent the forces that must be overcome by the energy released in homogenization, the degree of dispersion or the efficiency of the process increases, within certain limits, with increases in temperature. The temperatures used may vary, but in most commercial practices the process is carried out at, or near, the temperature of pasteurization, 63°.

Rahn[81] has studied the effect of homogenization at 20°, 40°, and 65° upon the distribution of the fat among groups of globules of various sizes. His results are given in Table LXXVII.

Table LXXVII.—Per cent of fat in each size group at different temperatures of homogenization. (From Rahn.)

Size of globules	Temperatures of homogenization		
	20°	40°	65°
μ	per cent	per cent	per cent
0-1	2.3	1.9	4.3
1-2	29.3	36.7	74.4
2-3	23.3	21.0	9.0
3-4	29.8	25.2	12.3
4-5	0.0	15.2	0.0
5-6	15.4	0.0	0.0

It is interesting to note the marked increase in the degree of dispersion with increases in temperature, especially between 40° and 65°. The surface tension of the milk decreases rapidly in this region, and at approximately 65° the ability of globules to agglutinate is nil.

The degree of dispersion depends also upon the rate of flow of the liquid through the homogenizer, this being in turn dependent upon the pressure used. Hatschek[41] has shown that as the sizes of the globules decrease the force necessary to subdivide them further increases enormously. Practical experience has shown that in order to produce homogenized milks of sufficient stability to prevent fat separation, pressures of 2,500 to 3,000 pounds per square inch are necessary.

The distribution of globules and their relative sizes in homogenized milks have been studied by Rahn.[81] These experiments were carried out upon milk prepared under conditions which conform closely to those used in industrial practice. Tables LXXVIII and LXXIX, from the results of these investigations, illustrate the fat distribution in skim milk and in homogenized whole milk.

Table LXXVIII.—Relative numbers of globules in skim milk and homogenized whole milk. (From Rahn.)

Globule diameter	Skim milk	Homogenized whole milks		
		I	II	III
μ	No.	No.	No.	No.
0-1	41.8	19.2	89.2	69.3
1-2	47.7	66.5	10.3	29.5
2-3	9.2	12.6	0.5	1.2
3-4	0.9	1.7	0.0	0.0
4-5	0.3	0.0	0.0	0.0
5-6	0.1	0.0	0.0	0.0
6-	0.0	0.0	0.0	0.0

Table LXXIX.—Relative amounts of fat in groups of globules in skim milk and homogenized whole milk. (From Rahn.)

Globule diameter	Skim milk	Homogenized whole milks		
		I	II	III
μ	per cent fat	per cent fat	per cent fat	per cent fat
0-1	1.7	4.8	4.2	1.2
1-2	40.4	43.0	81.5	83.6
2-3	30.8	37.6	14.3	15.2
3-4	13.4	14.6	0.0	0.0
4-5	8.0	0.0	0.0	0.0
5-6	6.0	0.0	0.0	0.0
6-	0.0	0.0	0.0	0.0

The fat remaining in skim milk consists mainly of globules approximately equally divided in number between two groups—those of less than 1 μ diameter and those of 2 to 3 μ diameter. Because of their extremely

small diameters, however, they represent less than one-half of the total fat content. In the homogenized whole milks the greater part of the fat is dispersed into globules of less than 2 μ diameter. It is evident, therefore, that homogenization carried out in the majority of practices renders the fat in a dispersed form more finely divided than the fat remaining in skim milk.

As indicated in a previous section, the smaller globules are less susceptible to forces of agglutination and coalescence. Their inability to clump readily, therefore, prevents their rising to form cream. (See creaming.) Von Sobbe [114] has studied the rise of fat in two homogenized milks allowed to stand for 72 hours. The amount of fat in the upper, middle, and lower 50 cc. of the cylinder were as shown in Table LXXX.

Table LXXX.—Distribution of fat in raw and homogenized milk after standing 72 hours. (From Von Sobbe.)

Sample	I		II	
Initial fat content	2.60 per cent		3.30 per cent	
	Raw	Homogenized	Raw	Homogenized
	per cent	per cent	per cent	per cent
Lower 50 cc.	0.30	2.3	0.20	2.95
Middle 50 cc.	1.40	2.5	2.50	3.20
Upper 50 cc.	8.50	2.9	14.50	3.85

Wiegner [121] has studied the degree of dispersion of milk fat upon homogenization at 250 atmospheres (approximately 3,750 pounds per square inch). Milk containing 3.13 per cent fat, whose globules were of 2.86 μ average diameter, contained globules of 0.27 μ average diameter after homogenization. Homogenization under these conditions, therefore, increased the number of globules 1,200 times and their surface area 117 times, and consequently greatly increased surface adsorption.

Emulsions consisting of globules of the sizes produced in these experiments are extremely stable and movements of the globules are determined almost entirely by their Brownian motion.

Separation of fat globules (spontaneous). The size of fat globules is a question of considerable interest and importance when viewed from the standpoint of their separation from the milk. Being of a specific gravity less than that of the medium in which they are suspended they tend to rise to the surface. The rate of rise of individual oil globules in a liquid medium may be calculated by means of Stokes' law for the rate of settling of spherical particles,

$$r^2 = 2/9 \frac{(d_1 - d_2)g}{N} \cdot V$$

which for the case at hand becomes

$$r^2 = 9/2 \frac{N}{(d_1 - d_2)g} \cdot V$$

or

$$V = r^2 . \frac{2(d_1 - d_2)g}{9N}$$

where r = radius of globule
d_1 = density of the dispersion medium
d_2 = density of the dispersed phase
N = viscosity of dispersion medium in absolute mass
g = acceleration of gravity
V = velocity of particle.

When dealing with the relative rates of rise of two globules in the same medium the expression $9/2 \frac{N}{(d_1 - d_2)g}$ becomes a constant (K), and V is proportional to r^2. The relative rate of rise of globules in the same medium varies therefore as their radius squared (r^2), or

$$\frac{V_1}{V_2} = \frac{(r_1)^2}{(r_2)^2}$$

K varies with the physical properties of the system as indicated in the formula.[97, 103]

From results of observations upon the rate of rise of 100 fat globules in a sample of milk Van Dam and Sirks [103] calculated the average size of globules in various groups by means of the Stokes formula. Their results agree surprisingly well with those obtained by direct microscopic observations of numbers and sizes. These authors also measured the rate of rise of a globule 3.3 μ diameter in three milks and found the average rate to be 0.18 cm./hr. The results indicated, however, that individual milks vary greatly with respect to the rate of rise of globules of the same dimensions.

Addition of 0.75 per cent of gum tragacanth to the milk reduced the rate from 0.18 cm./hr. to 0.10 cm./hr. According to Stokes' law the viscosity change due to this added substance is not great enough to account for the noted change in velocity of rise. Specific adsorption and hydration changes at the globule/serum interface must therefore exert marked effect upon the rates of rise of globules, and perhaps explains the marked variations in some milks with respect to rates of globule rise. (See Cream rising.)

The rate of rise of a globule of 3.3 μ diameter calculated from the data of van der Burg [104] is of the order of 0.17 cm./hr., which is in good agreement with the value obtained by Van Dam and Sirks.

The results obtained by Rahn [80] indicate that the rates of rise of individual globules of small dimensions vary considerably, and also that their rates of rise are slightly greater than would be expected.

Table LXXXI illustrates the rates of rise of globules of different diameters according to the data of these authors. The theoretical values calculated according to Stokes' law from the values of Van Dam and Sirks are also included. Values other than those designated were calculated from the experimental value in each case, through the relationship $\frac{V_1}{V_2}=\frac{(r_1)^2}{(r_2)^2}$.

Table LXXXI.—The rates of rise of globules of different diameters.

		Van Dam and Sirks		Van der Burg	Rahn
Diameter	Radius	V (Theor)	V	V (calc.)	V (measured)
μ	μ	cm/hr.	cm/hr.	cm/hr.	cm/hr.
2	1	0.051	0.066	0.062	
3.3	1.65	.14	.18 (measured)	.17	0.12-0.25 (av. 0.185)
5	2.50	.32	.41	.39	0.22-0.35 (av. 0.285)
10	5.00	1.28	1.64	1.56	0.95

Allowances must be made for variations between individual milks, for slight errors in the measurement of values of the order of magnitude dealt with here; as well as for viscosity factor corrections, the numerical values of which are not known.

Troy and Sharp [102] have measured the rates of rise of globules varying widely in size and their results illustrate the validity of the use of Stokes' law in calculations of the rise of fat globules in milk.

In Figure 16 are given their experimental values obtained at 24°.

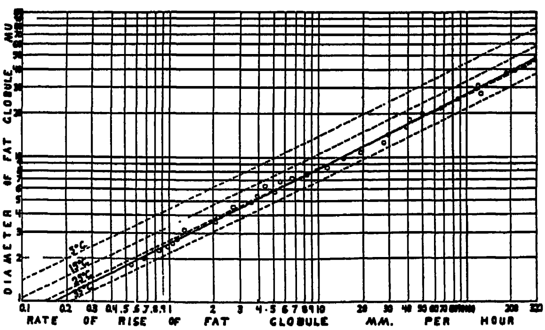


FIG. 16.—Rate of rise of fat globules as affected by the size of the globules. (From Troy and Sharp.)

The broken lines represent the values at the different temperatures, calculated according to Stokes' law.

The agreements between the experimental and calculated values are excellent. These values also agree well with those obtained by Van Dam and Sirks and by van der Burg.

It is obvious from the figures given, however, that the rate of rise of individual globules is not rapid enough to account for the rapid formation of cream layers upon milk. This phenomenon must, therefore, be explained upon the basis of the fact that globules aggregate to form clumps whose rate of rise is relatively rapid.

Troy and Sharp measured also the rates of rise of spherical clumps at 25°. The broken lines in Figure 17 represent the values for the rates of rise of clumps of the assumed fat content designated.

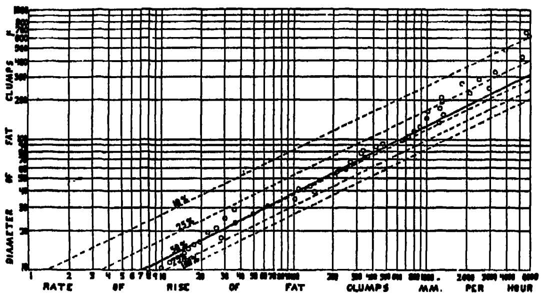


FIG. 17.—Rate of rise of fat clumps as affected by the size of the clumps. (From Troy and Sharp.)

The results indicate that clumps are of approximately 50 per cent fat content and that their rates of rise are rapid enough to account for rapid cream rising. The deviation of their results from the calculated values in the case of large clumps is due to a retarded rate of rise caused by the resistance offered by the walls of the chamber.

Cream rising. Since the rapid formation of cream layers upon milk must be accounted for through the rise of aggregates which form as the globules come into contact with one another, the rate of creaming is dependent upon the extent of aggregation, and the condition favoring most rapid aggregation promotes the most rapid creaming.

Microscopic examinations of milk show that the clumping tendency is greatest if the milk is cooled rapidly to from 7° to 8°.[108] Practical experience has shown also that temperatures in this region are the most favorable to rapid cream rising.[92, 108] The physical state of the fat when creaming takes place also influences the rate. If the milk is held at a low temperature, until the fat becomes solid, before creaming is allowed to take place, the rate is retarded considerably. Best results seem to be obtained with a medium of low temperature and the fat in a semisolid condition. Slow cooling to a temperature slightly above the melting point of the fat and rapid cooling to the temperature at which creaming is to take place seems to give the best results.

It is also true that conditions that are optimum for clumping produce

the greatest cream volume. This is most easily explained upon the basis of "piling up" of clumps. The large clumps produce the larger interstices and hence will occupy the greater volume. Smaller clumps permit of closer packing and hence occupy less volume. The results of too high creaming temperatures are evident. Clumps of low plasticity deform readily and hence result in decreased volume through closer packing.

Though the apparent cream volume is not necessarily an index of the amount of fat which has risen as cream, it is generally true that most complete creaming is associated with the greatest cream volume. Since the rate of cream formation and the apparent amount formed are of great importance commercially, some of the conclusions from a large number of experiments by Whittaker, Archibald, Shere and Clement [117] are given here:

"Milk heated to 143° F. for 30 minutes showed practically no decrease in the cream volume, and in some cases an increase resulted.

"Pasteurization at 145° to 146° F. for 30 minutes reduced the cream volume an average of approximately 8 per cent, with considerable variations above and below.

"The reduction in cream volume due to pasteurization at 145° to 146° F. was not so marked in the case of 24 to 36 hours old milk as in fresh milk, owing to the fact that old milk does not show so deep a cream volume before pasteurization as does fresh milk. Pasteurization of old market milk had a tendency to restore its creaming ability.

"The tests to show the effect of agitation during the holding period were not uniform, but the tendency appeared to be slightly in favor of holding the milk without agitation during this period. Moderate agitation during the 30-minute period apparently has no appreciable effect on the cream volume.

"The tests showed that cooling milk to a low temperature after pasteurization is necessary in order to obtain a good cream volume.

"Pasteurized milk cooled to below 45° F. showed a much better cream volume than that cooled to only 50° F. or above.

"Milk stored at 38° to 48° F. showed a much better cream volume than that stored at temperatures above 50° F. The difference in favor of the colder storage temperature was more marked in the case of the raw milk than in that of the pasteurized milk.

"The cream volume of milk pasteurized at temperatures from 143° to 145° F. was greater after two or three hours' storage at low temperatures than in the original raw milk. This gradually decreased as the storage period was prolonged.

"The recreaming of raw milk decreased the cream volume, but after one recreaming the age of the milk was of more importance than the number of times it was recreamed.

"The recreaming of pasteurized milk had a detrimental effect on the cream volume. This had a practical significance at plants which bottle pasteurized milk after it has been allowed to cream.

"Allowing milk to stand or to be agitated for 15 minutes or more at temperatures between 60° and 110° F. had generally a detrimental effect on the cream volume. This was more pronounced with milk that was being cooled from the pasteurization temperature than with that which was being heated.

"Clarifying milk at temperatures between 60° and 65° F. had only a slight effect on the cream volume, though there was a considerable decrease when the milk was clarified at temperatures above 80° F."

Dahlberg and Marquardt [26] have also shown that pasteurization at temperatures of 140° to 145° F. for 30 minutes does not greatly affect the cream volume. However, at higher temperatures there is a marked reduction in the volume.[57] They give the following relationship between time and temperature of heating and minimum reduction in cream volume.

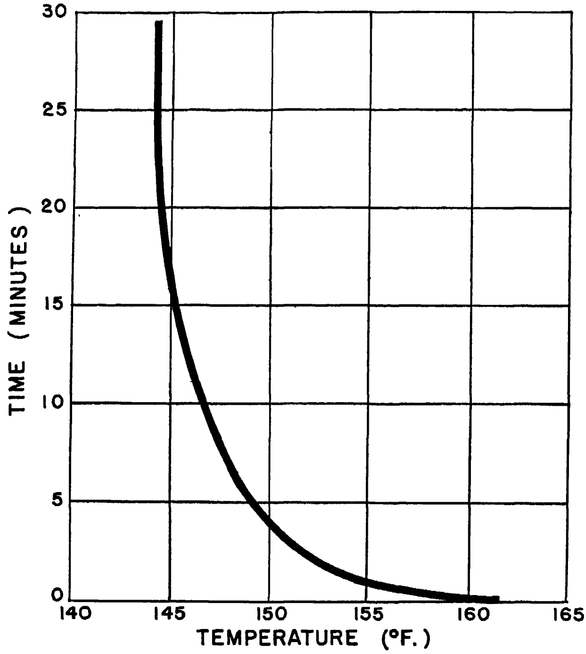


FIG. 18.—Length of time that milk can be held at various temperatures with the smallest reduction of cream layer volume.—From Dahlberg and Marquardt.

On the basis of these results they conclude that for securing pasteurized milk of maximum cream volume, from a milk of given fat content, pasteurization should be carried out at a temperature slightly less than 145° F., for a period of 30 minutes. Further, the fat globules of the original milk should be retained in their normal condition by prevention of excessive churning, agitation, freezing or oiling off; and following pasteurization the milk should be cooled to 40° F. or below and bottled immediately. After bottling it should be held at 40° F. or below, since the cream volume shrinks markedly at 50° F. or above.

However, various milks treated similarly may show different creaming properties. In these cases the variations in properties or forces active at the globule surfaces must be concerned.

Van Dam and Sirks [103] and Palmer and Anderson [67] studied the factors influencing cream rising and concluded that the milk plasma primarily influences creaming. Rahn [78] has drawn similar conclusions and believes that the active substance in the aggregation phenomenon is a substance similar in nature to the "slime" described by Storch.

The experiments of Hekma [48] are significant and suggestive as to the chemical nature of such a substance. His results with blood globulins indicate that substances of this type, especially of the euglobulin type, are very active in the agglutination phenomenon of globules.

In addition to the agglutin phenomena as a basis for the explanation

of clumping Dahlberg and Marquardt [26] postulate that the magnitude of the charge carried by the fat globules is of importance in determining the ease with which aggregation occurs. Fat globules carry negative charges which when partially neutralized by the strongly positive ions decrease electrostatic repulsion between globules and hence favor aggregation. This view was employed by these authors to explain the difference in creaming properties of different milks similarly treated and the beneficial effect of the heating of milk upon creaming.

Through the addition of various hydrophyllic colloids to milk (gum tragacanth, gelatin, starch, gum arabic, agar, etc.) the creaming ability may be greatly increased and the cream volume may be increased 15 to 20 per cent. The viscosity may increase 25 to 100 per cent. Palmer and Anderson [67] maintain that the specific viscosity of milk is due largely to the plasma colloids. They have correlated this property with creaming ability and maintain also that the viscosity of a raw milk is a good index of this property and can be used as an explanation of changes in cream layers caused by the temperature of creaming or the plasma colloids in milk. The effect of pasteurization can not be explained by viscosity changes, according to these authors.

Rahn [79] has noted a similar correlation between viscosity and creaming ability but concludes that variations in creaming are not due to variations in viscosity.

Though the viscosity is usually of a greater value in the cases which show enhanced creaming ability there seems little doubt that in most cases the degree of exhibition of this physical property is a result of the physical states rather than a reason for and explanation of their cause. The "increased viscosity" which has been noted in many cases is undoubtedly plasticity or the resistance offered by the larger aggregates present in the one of greater viscosity. This property may therefore be regarded largely as a manifestation of macroscopic structure brought about by favorable conditions at the interfaces. The ultimate explanation must lie in an explanation of the conditions and of the forces acting at the fat/skim milk interfaces. This involves the study of the specific nature of substances in milks as well as their adsorption equilibria at the various temperatures.

Separation of fat (mechanical). Under commercial conditions the rate of separation of the fat from the skim milk must be increased greatly over that of normal cream rising. This is accomplished in the bowl of the separator rotating at a speed of from 3,000 to 16,000 R.P.M., the speed depending upon its construction. Under these conditions gravity is increased to 1000 times or more its normal value and, as the expression $g(d_1 - d_2)$ in Stokes' law increases in value, that of K decreases.

$$r^2 = KV \text{ or } r^2/K = V$$

or, the velocity increases are proportional to the decreases in the value of K.

The efficiency of separation depends also upon the length of time that this increased centrifugal force acts. The extent to which this factor can

be increased is limited by capacity considerations. However, even under the increased gravitational force the distance traveled by a fat globule of the average size in milk during the time it remains in the separator is of relatively small magnitude. .To facilitate separation, therefore, the bowls are so constructed that the distance the globules must travel is minimized as much as possible. This is usually accomplished through the arrangement of from 20 to 40 A-shaped discs within the bowl, of the general arrangement shown in Figure 19. This mechanical arrangement reduces the maximum distance of travel of the fat globules to that of the horizontal distance between the discs. When the globules reach the discs the smaller ones are carried along by the larger ones and by the clumps.

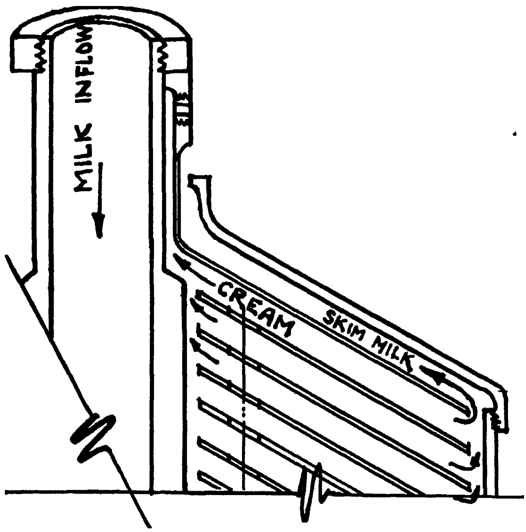


FIG. 19.—Diagrammatic cross-section of a cream separator bowl.

Even under this great increase of gravity the rate of travel of the smallest globules can not be increased to the extent that a separation can be effected in the short time which the milk remains in the bowl of the machine. Those globules whose rate of motion is less than the rate of movement of the stream of milk passing to the outside, will be swept out mechanically and will appear in the skim milk.

Rahn [81] concludes from his studies upon the distribution of fat in skim milk that globules of less than 1 μ diameter are not removed during separation. Those of 1 to 2 μ diameter are but slightly affected and are present in about the same numbers in skim milk as they are found in the original whole milk. Those of 2 to 3 μ diameter are found mostly in the cream. Of those greater than 3 μ diameter practically all are found in the cream.

The results obtained for the distribution of globules in whole milk, skim milk, and cream have been given in Table LXXVI.

Foaming. A foam consists of a gas phase (usually air) dispersed in a liquid phase. The gas phase is in the form of bubbles of microscopic sizes. Under these conditions an air phase of enormous surface area is produced over which are extended films of micronic or submicronic thickness.

In order that these films may form, the surface tension of the liquid phase must be sufficiently reduced to allow the gathering and spreading of the active agent into thin films. However, a low surface tension alone is not sufficient for producing a stable foam.[44] The films or lamellae must be sufficiently elastic and tough to prevent coalescence of the air bubbles. Thus, if the surface tension of the liquid is not great enough to withdraw the film from between the globules, and if the stabilizing agent has great internal viscosity, conditions are favorable for the formation of a stable emulsion.[75]

It has been pointed out by Holmes and Child,[45] and by Clark and Mann [19] that the maximum viscosity obtainable is not necessarily the optimum viscosity desired for the best emulsification properties. It is evident therefore that the stability of a foam is largely dependent upon the peculiar specific properties of the films. Other properties, secondary in their nature, are known to be concerned in the formation of stable foams, namely,—thickness and vapor pressure of the films, and the presence of finely divided materials at the interfaces.[36, 21a]

The tendency of milk to foam when agitated is evidence of the presence of a surface tension depressant,—in this case proteins. With increases in the fat content of a milk or cream the surface tension is further reduced. This occurs as a natural consequence of the laws governing adsorption, since the material surrounding the fat globules must be composed of those substances which lower the interfacial tension to the greatest extent. Hence, the addition of fat globules decreases the interfacial tension. Changes in protein concentration should also be considered for, as shown by Behrendt,[11] the surface tension value is critical to these variations. Burri and Nussbaumer,[16] and Bauer,[8] showed that the surface tension of milk decreases upon aging and is lowered abruptly when milk is cooled. These authors, as well as Quagliariello,[76] associated this phenomenon with solidification of all fat, the latter author believing that the lower soluble glycerides enter into this phenomenon. These experiments indicate a dynamic surface tension possessing a slow rate of change, thereby denoting that the orientation of those fractions of protein possessing the property of reducing the surface tension to the greatest degree is a slow process. These and other results indicate that the phenomena dealt with are the results of a shifting adsorption equilibrium.

Between the temperatures of 20° and 30° the tendency of the milk to foam is at a minimum.[52] Below this range foaming increases with decreases in temperature. Above 30° there is a rapid increase in the foaming tendency with increases in temperature. Figure 20 illustrates the instability of the foams produced at the lower temperatures as well as the shifting of the region of minimum foaming to higher temperatures

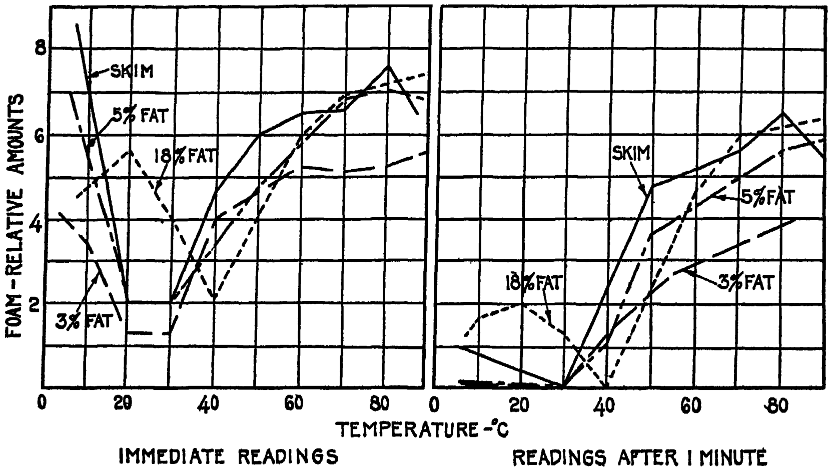


FIG. 20.—The foaming tendencies of milk and cream at different temperatures and the stability of the foams produced. From C. S. Leete.

with milk of increased fat content. Pasteurization reduces slightly the foaming tendency at the various temperatures.

An attempt to correlate the surface tension of milks with their foaming properties at various temperatures, indicates that, though there is a general correlation, this property alone does not account for the results obtained. At temperatures below 20° the surface tensions of milks are approximately constant at 56 to 59 dynes/cm. As the temperature is increased the magnitude of the surface tension decreases slightly. Between 45° and 60° the value lowers more rapidly to approximately 42 to 45 dynes/cm.

The values given in Figure 21 must not be accepted as absolute values representative for all milks, but only as indicative of the general trend of variations of surface tension with those of temperature. The values are those determined immediately after the various temperatures were attained. The final values at equilibrium were not determined. Individual samples vary with respect to this property and each sample varies greatly with previous treatment. The lower surface tension in the region of higher temperatures allows for greater adsorption of the protein in the air/liquid interfaces, and results in a decrease in the interfacial energy as well as a decrease of the forces exerted by the films. Though these facts contribute to the explanation of the tendency to foam at higher temperatures, they do not explain the minimum foaming tendency at 20° to 30° and the increase of this tendency below these temperatures.

Foams are labile structures and a distinction must be made between foaming tendency and the stability of the foam produced. The tendency of milks to foam at the lower temperatures is undoubtedly a result of variation in those properties of the solution which evidence themselves in viscosity changes. The instability of these foams indicates that they are highly hydrated and possess high vapor pressure. The films are undoubt-

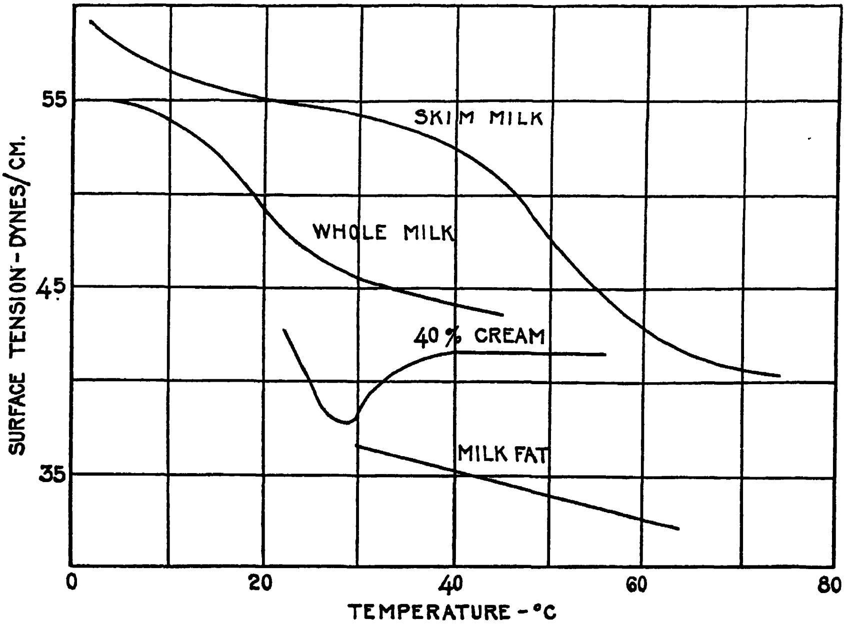


FIG. 21.—Effect of temperature change on the surface tension of milk, cream and milk fat. (Author's unpublished data, 1927.)

edly of great thickness and, therefore, according to Poiseuille's law, drain rapidly.

At the higher temperatures drier and thinner films are formed, which are stable. This stability is further increased through the denaturization of the proteins in the films.[84] This action renders them rigid and thus increases structural stability. On the basis of the theory that at the lower temperatures the hydration of the constituents is materially increased, the region of minimum foaming (20° to 30°) represents the zone in which this effect is practically nil, and 30° represents the temperature at which the other factors begin to exert their predominating effects.

Large increases in the fat content of the milk over those normally present not only increase foaming but also stabilize the foam produced. In addition the region of minimum foaming is shifted to 40°. The presence of normal fat globules above a certain concentration has undoubtedly some stabilizing action upon the foams formed. The "piling up" effect, solidity of the fat, method of treatment of the system and other conditions must be considered here in addition to those factors already mentioned if an explanation is attempted.

The data upon the relation of viscosity to foaming deal mainly with viscosity in its relation to cream whipping, and fail to distinguish between structural effects and true viscosity. These data are, therefore, of little value in the study of conditions favoring film formation, since they are a result of the physical state rather than a reason for its existence.

Cream whipping. Whipping of cream consists essentially of beating air into cream to form a stable foam. Although increases in the fat content of creams reduce their surface tensions and increase their foaming tendencies, the slight variations in this property do not account for the great variations in whipping quality. The main factor concerned is that of the stabilizing action of the fat globules and their aggregates.

During the whipping process air is incorporated in the cream and the fat globules and their aggregates gather in the liquid/air interfaces, or films, and produce a "piling up" effect which results in a rather rigid structure.

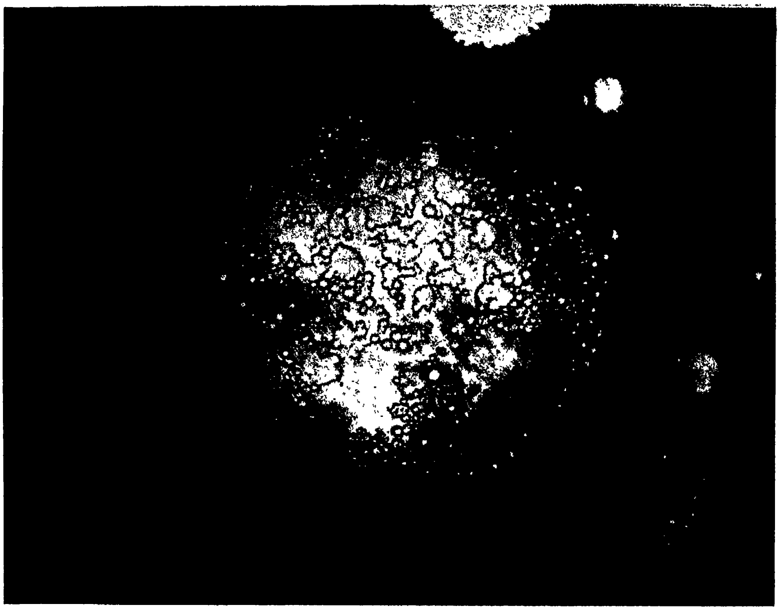


FIG. 22.—Photomicrograph of whipped cream, showing a large air cell and the irregular fat clumps which interfere with the fluidity of the cream. (From Dahlberg and Hening.)

Simultaneously with the stiffening of the cream, the fat globules show distortion and clumping analogous to the beginning of a churning process. That this is actually a partial churning is shown by the fact that additional whipping beyond the point of maximum stiffness results in butter formation.

The added stability produced by the presence of the fat aggregates allows for greater subdivision of the air phase thereby producing a great extension of its surface area and of the protein films. This results in thinning of the lamellæ to the point where drainage is practically nil, and hence a dry foam results. The thinning of the films is aided by increases in the fat surface over which the protein film must spread. An additional fact that contributes to the stability of the foams produced is that of

denaturation of the proteins whereby are produced rigid films of mechanical firmness.

To obtain a "piling up" effect it is necessary that the fat be in a solid or semisolid state. However, individual globules do not pile up readily to form rigid structures and, therefore, a slight churning or a clumping effect seems necessary. With a lowering of the temperature of the cream both conditions are attained. Aggregation and solidity of the fat are increased.

The relation of temperature and percentage of fat content to whipping ability has been shown by Babcock[8] and by Dahlberg and Hening.[25] These results indicate that maximum whipping efficiency may be obtained

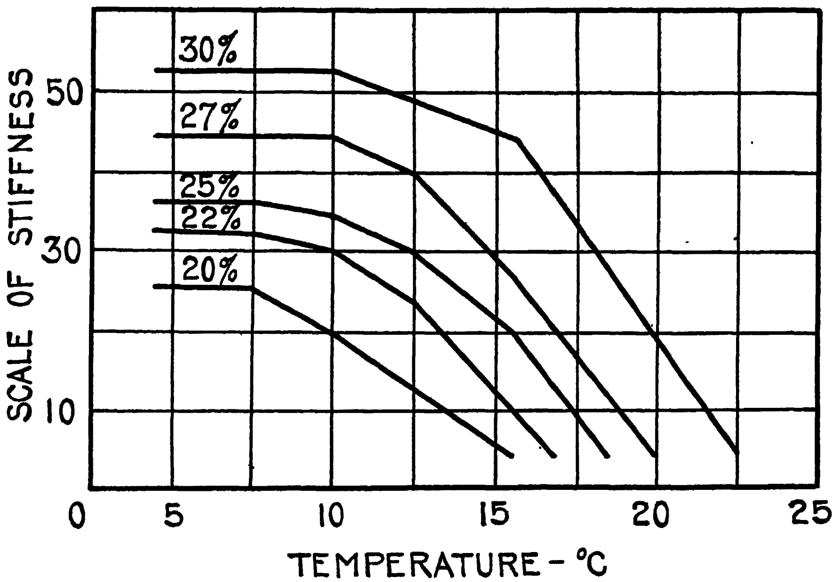


FIG. 23.—Effect of temperature on whipping quality of cream. (From Babcock.)

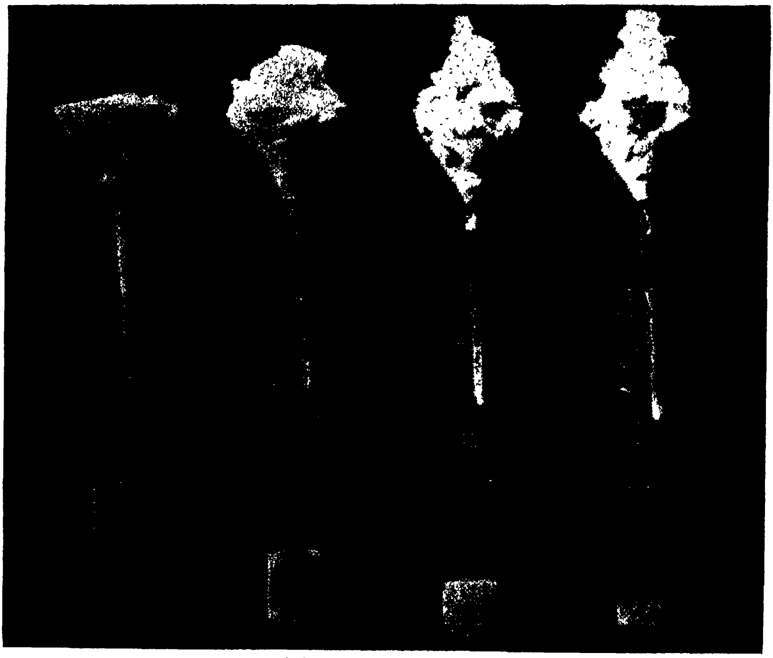
at temperatures closely approximating those at which clumping is most rapid. The effect of increased fat content is also illustrated in the following figure taken from the report of the work of Dahlberg and Hening. Increase of the fat content produces increased stiffness and minimum drainage of the whip.

The various processes that may be used in the handling of cream affect its whipping quality through variations in the degree of dispersion and the ability of the globules to form clumps.

Pasteurization disperses the fat aggregates and also partially destroys the clumping ability; and therefore has a detrimental effect upon whipping ability. The following results of Dahlberg and Hening and those of Babcock (see Figure 23) illustrate the superiority of raw cream over pasteurized cream for whipping purposes.

The results in Figure 25 illustrate also the effect of globule sizes. Large globules agglutinate more readily than do those of lesser dimensions, thus favoring structural formations to a greater degree. The difference shown here in whips produced with creams from Jersey and Holstein milks is therefore probably primarily a result of differences in globule sizes.

Homogenization destroys almost entirely the ability of fat globules to agglutinate. Increases in the pressures used increase subdivision of the



20% 25% 30% 35%

FIG. 24.—Whipped raw cream, with different percentages of fat. Holstein cream, whipped after aging. (From Dahlberg and Hening.)

fat which further decreases ability to form foam structures. The effect of variations in this factor is shown in Figure 26.

Clayton,[21b] and Palmer [72] explain this effect by assuming that in the homogenized state the fat globules have adsorbed the protective proteins in the solution to such an extent that the amounts which remain are not sufficiently large to permit of adsorption at the liquid/air interface,—i.e., to stabilize the foam. Added proteins of a protective nature restore the whipping quality.

Aging of cream is conducive to clumping and, therefore, has an effect opposite to those of pasteurization and homogenization. The detrimental effect of pasteurization and the enhancing effect of aging upon whipping quality is shown in Figure 27.

The effect of the temperature of the milk at the time of separation of the cream is an important factor in regulating the body of the product. When the fat globules of the raw cream are in the liquid condition at the time of separation they are dispersed in the serum of the cream and

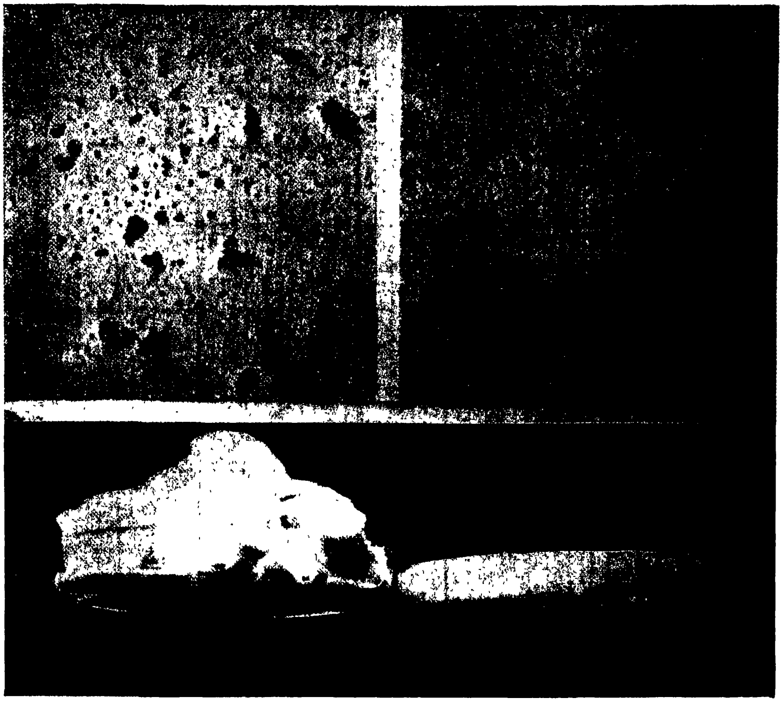


FIG. 25.—Above, Photomicrograph of fat globules and clumps in cream.

Upper left, fat clumping in high viscosity cream produced by separating milk at 26° C. after previous cooling of the milk long enough to harden the fat.

Upper right, individual fat globules in low viscosity cream produced by separating milk at 26° C. immediately after milking or pasteurization.

Below, Cream of variable viscosity.

Two samples of cream from the same milk, each with 35 per cent fat; that on the left separated to produce high viscosity, that on the right to produce low viscosity. (From Dahlberg and Hening.)

produce a cream of low body which increases but slightly upon aging. If the fat is in a semisolid condition during separation, clumping is promoted and a cream of heavy body results. Such a cream upon aging becomes very plastic. Similarly Dahlberg and Hening have shown that the globules in cream from pasteurized milk can be made to clump and the cream to become viscous upon aging. (Figure 28.)

It is evident that the state of subdivision of the fat phase is of prime importance in relation to whipping quality of a cream.

As heretofore stated, many of the results of viscosity studies upon cream are measurements of the structural properties of the systems (plasticity) and are therefore contributory to the knowledge of this state and are not a measure of true viscosity. The increase of body of creams upon homogenization is due to volume increases of the fat phase through increased adsorption and must, therefore, be excepted. On the contrary the addition of sugar, which increases the true viscosity, decreases whipping quality.

Viscogen, like many protective proteins, greatly enhances aggregation

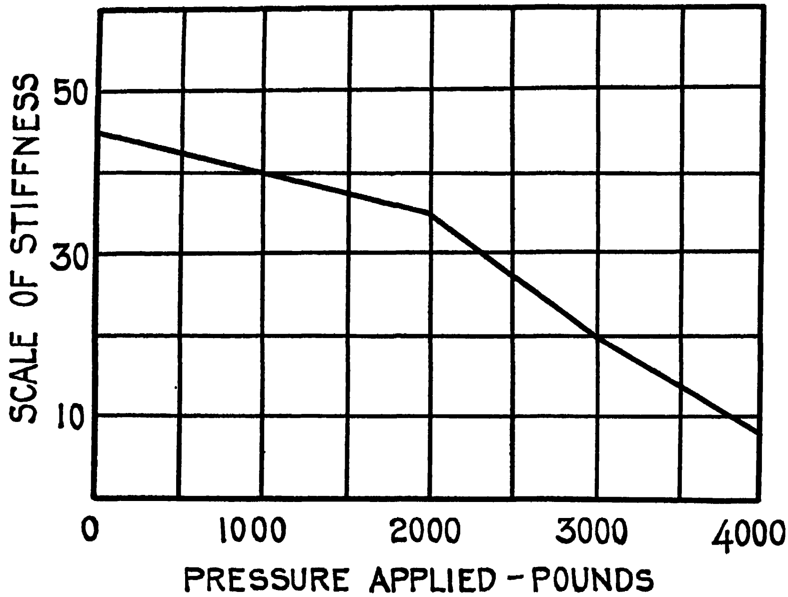


FIG. 26.—Relation of homogenizing pressure to whipping quality of 30 per cent cream. (From Babcock.)

and whipping quality. Other factors seem to be involved when this agent is used though no attempt at an explanation has been made.

Increases in acidity of the cream have practically no effect upon the whipping quality.

When cream is powdered the whipping ability is destroyed entirely. This results, undoubtedly, through denaturation of the proteins in the drying process.

Churning. Continued agitation of cream, held at temperatures that are favorable to the aggregation of fat globules, results in a relatively rapid progressive growth of clumps. When the ratio of the surface area of the clumps to their cubical content becomes relatively small the emulsion breaks and the fat gathers in the form of a plastic mass—butter. The rate at which this phenomenon proceeds is primarily dependent upon the temperature but is dependent also upon other factors (see pp. 176

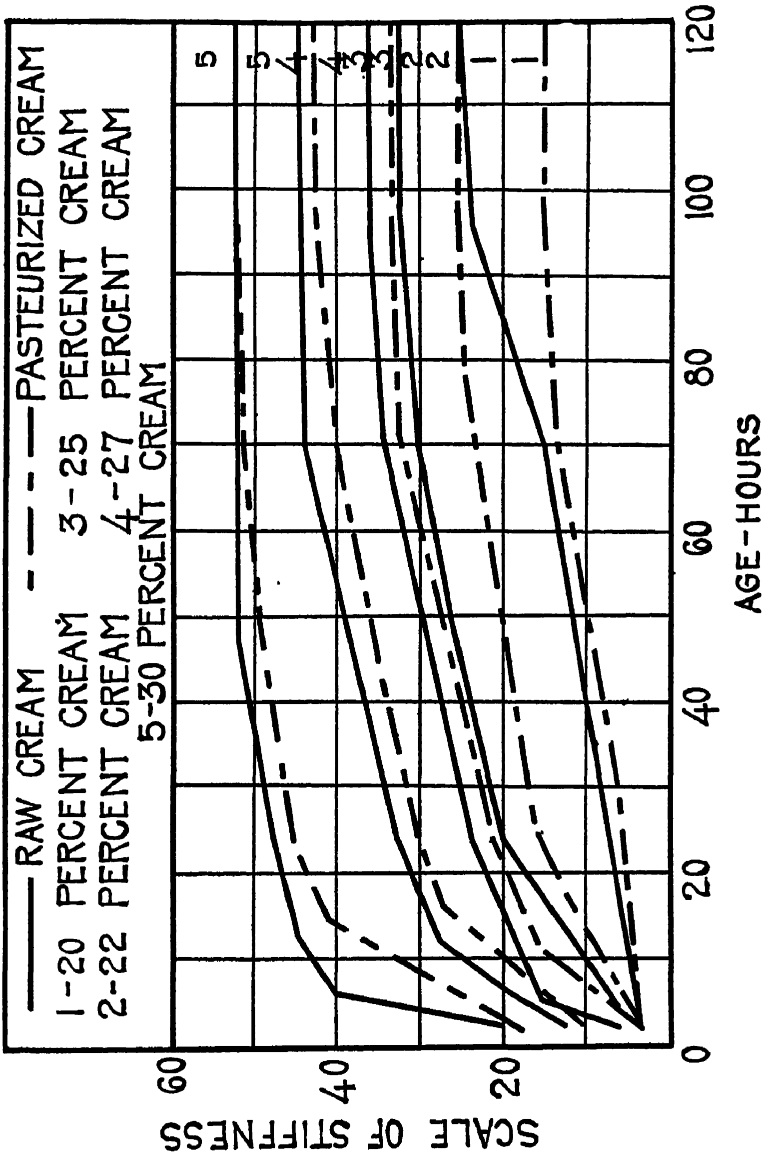


Fig. 27.—Effect of pasteurization on whipping quality of cream. (From Babcock.)

and 178). When air is blown through milk the fat may be recovered in the foam; [22] indicating that the fat globules aggregate in the air/serum interfaces. Air beaten into the cream during churning serves, therefore, as a medium for bringing the globules into contact, thereby enhancing the rate of aggregation. Because of their greater stability the small air bubbles are more effective in this respect than are the larger ones. Those sufficiently minute to assure stability throughout the process become a part of the aggregates and are incorporated into the mass of butter. Small water droplets, stabilized by protective films and by fat globules

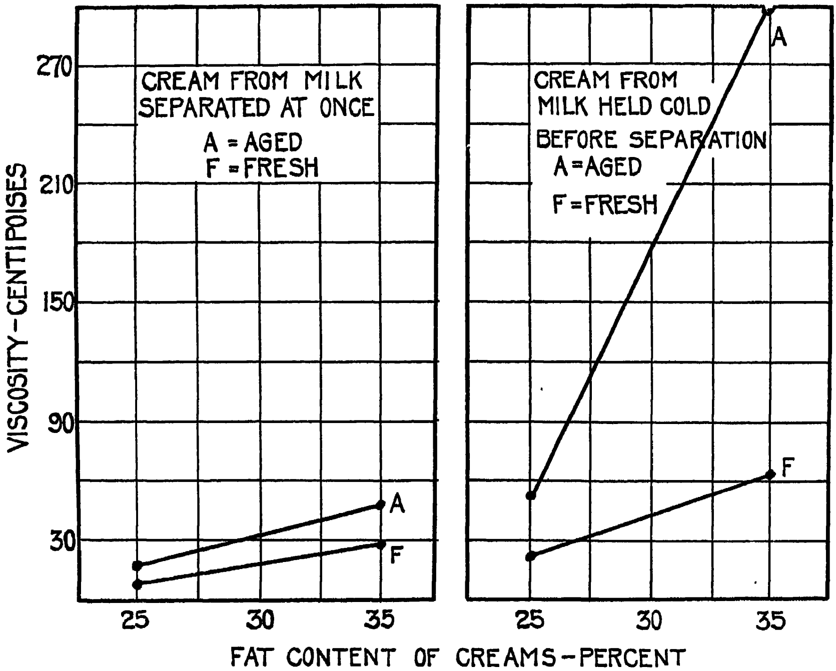


FIG. 28.—Influence of the condition of the fat in pasteurized milk at the time of separation upon the viscosity of the resulting cream. (From Dahlberg and Hening.)

which collect in the interfaces, are also incorporated into the butter. Butter consists, therefore, of fat globules, air bubbles and water droplets each surrounded by protective films, and dispersed, as it were, throughout a mass of free fat.

In the report of a series of excellent studies upon the structure of butters Storch [96] gives the numbers and sizes of these water droplets as follows:

	Number per cu. mm.
Less than 10 μ diameter	2,000,000-13,000,000
Over 10 μ diameter	4,000- 6,000

In addition to the droplets, water is also occluded when the large aggregates are joined.

The distribution of water droplets in butters according to numbers and sizes has been studied also by Boysen [13] who gives the figures of Table LXXXII.

Table LXXXII.—Distribution of water droplets in butter according to numbers and sizes. (From Boysen.)

Average diameter of droplets	Number of droplets
μ	per cc.
1.90	12,905,730
4.00	230,490
7.50	61,469
12.50	10,740
20.00	628
30.00	130
40.00	57
50.00	25
60.00	12
70.00	8
80.00	5
90.00	4

The temperatures at which cream will churn satisfactorily cover a relatively narrow range, namely, 10° to 24°. At temperatures above 24° and below the melting point of the fat, little butter is obtained, and that obtained is usually of poor quality. Above the melting point, no amount of churning will produce butter, but there is produced a subdivision of the large globules. Below 4° the globules do not adhere to one another and therefore churning of cream held at or below this temperature merely distorts the shape of the globules and causes no aggregation.[4a]

Two theories have been advanced for the formation of butter from cream during churning. Fischer and Hooker [32] propose the theory that during the process there is a reversal of the fat-in-water emulsion to that of a water-in-fat emulsion. Rahn [82] has presented the other view held by many, namely, that butter formation is mainly a packing of the globules into a compact mass and that the water and air are enmeshed during the agitation. The latter author objects to the phase reversal theory on the ground that the formation of butter can not be a complete phase reversal because the fat is in a solid state.

The difference in the two theories lies mainly in the matter of point of view and of definitions. Two pure non-immiscible liquids do not form stable emulsions of a highly dispersed phase concentration. To accomplish this result a third or stabilizing phase is necessary. Considered from the standpoint of the relationship of the concentrations of the phases milk is a fat-in-water emulsion and butter a water-in-fat emulsion, each made possible by the stabilizing action of the protecting phase. In the latter product the fat phase is not continuous in a strict sense but exists in the form of globules. The only strictly continuous phase is that of the hydrated protein, or emulsifying agent, which is also continuous in

milk and cream. However, as Fischer and Hooker have pointed out, the formation of an emulsion is a mechanical process and its stabilization is a wholly different problem.

In the early stages of the churning process there is a progressive growth of clumps, or a packing of globules as maintained by Rahn. If the temperature of churning is unfavorably low this process may be continued until a mass of fat gathers. This mass is of whitish appearance and, when held at a temperature above that of the melting point of the fat, forms a heavy liquid resembling cream, without a separation of free fat. Though "butter" formed in this manner is not the butter of trade its properties indicate the first process concerned in butter formation, namely a packing of globules.

When the "break" occurs in butter formation the reaction is undoubtedly of a wholly different type. It is undoubtedly accompanied by a partial inversion of phases, since butter contains part of its fat in a free form. It seems, therefore, that both theories are applicable and are necessary to a complete explanation of how the final product is produced from cream.

The exact mechanism and the forces involved in the process are still in doubt. The constancy of the ratio of water to fat in butters churned under identical conditions indicates that there is a definite surface relationship between water droplets and fat globules. This would indicate that a definite surface area of the water phase is necessary to break the fat-in-water emulsion. As agglutination or clumping occurs there is a progressive inversion of the phases, and since aggregation proceeds at a geometric rate the "breaking" is apparently very rapid.

Palmer,[66] employing the methods of Bhatnagar for the study of emulsions has measured the electrical conductivity of cream during churning. The conductivity decreases (resistance increases) to a constant minimum. When the butter "breaks" the resistance drops to its original value. Rahn has shown that the same phenomenon may be observed when skim milk is churned and maintains that incorporation of air is a factor in increasing the resistance during churning.

The interfacial potential of the globules of an emulsified oil is an important factor in their stabilization. The results of Palmer seem to indicate that this factor is not of great magnitude in creams.

It is obvious from what has already been stated concerning clumping that as low temperatures as possible within the churning range are advisable. Churning at the higher temperatures results in a loss of fat, and an incorporation of increased amounts of water. If the temperature of churning is too low the granules form slowly, pack closely, and retain a small amount of water. Because of the fine divisibility of the water the conditions of excess moisture are not subject to correction. The condition of less moisture than is desired may be remedied by working the butter in water.

Working of butter frees the occluded water and tends also to incorporate and subdivide the droplets.[18] Excess working may cause subdi-

vision to the extent that the color of the product may be injured. The experiments of Storch and those of Boysen indicate clearly that dispersion of the water droplets is responsible for "whiteness" or mottled conditions of butter. The figures of Storch are given in Table LXXXIII.

Table LXXXIII.—Dispersion of water in butter.

Classes	Average number of droplets in 1 cu. mm. of butter				
	I	II	III	IV	V*
In clear part of mottled butter	138	371	979	4,498	2,832,500
In whitish part	55	229	574	4,262	10,950,000

* Class V represents the droplets of smallest size.

The effect of salting upon the distribution was also studied by Boysen who found that the water content was materially reduced. The reduction in the number of droplets was most pronounced in those of smallest diameters.

The necessity of proper temperatures in churning is evident. This factor must be adjusted to meet the conditions as determined by the properties of the milk fat. According to Hunziker [47] the temperature of the cream should be so regulated that churning is completed in from 40 to 50 minutes. The temperatures given by this author are 9° to 12° for cream from milk produced during the summer months and 13° to 15.5°

Table LXXXIV.—Distribution of water in sweet and salted butters. (From Boysen.)

Droplet sizes	Sweet butter		Salted butter	
μ	Number	per cent H_2O	Number	per cent H_2O
1.90	17,175,400	6.47	11,066,800	3.97
4.00	317,540	1.12	180,700	.61
7.50	72,080	1.67	60,230	1.33
12.50	9,040	.97	11,570	1.18
20.00	620	.27	600	.25
30.00	115	.17	160	.23
40.00	46	.16	69	.23
50.00	20	.14	29	.19
60.00	8.5	.10	16	.18
70.00	6	.11	12	.22
80.00	2.5	.07	6.5	.17
90.00	2.5	.10	5.1	.19
Over 100	...	10.28	...	3.61
	17,600,000	21.63	11,300,000	12.36

during the winter months when the melting point of the fat is somewhat higher.

Other factors concerned are viscosity of the cream, its fat content, and its acidity. Acidity decreases viscosity, allowing for greater freedom of

movement of the globules and hence greater chance of collision. Acid creams therefore churn more easily and more completely than do sweet creams. Increased concentration of fat globules results in more rapid aggregation and butter formation but promotes coalescence and numerous larger and easily disrupted globules, thus producing butter possessing a greasy body. The most suitable butterfat content of cream to be used for churning purposes is 30 to 33 per cent.

That variations occur in the churning ability of cream from milks produced by individual animals is well known. The globules differ in their agglutinability because of their sizes and also because of specific differences in the substances at their surfaces. Globules of large diameters clump readily and hence churn easily. This explains why cream from milks of Jersey and Guernsey cows churns more readily and more completely than does cream from the milks of other breeds.[4]

With advances in the lactation periods, the globules decrease in size and the time of churning generally increases.[106c] Late in the lactation period the fat globules become very small and churning becomes at times difficult and inefficient.

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Chapter VIII

Coagulation of Milk

Heat Coagulation

Denaturation and agglutination. The coagulation of proteins by heat consists of two reactions, denaturation and agglutination.[6, 35, 36, 37, 76, 102, 103, 104, 105] The two phenomena are well illustrated by experiments with egg albumin. Salt-free albumin in solution will not coagulate on boiling, but will be denatured and, after such treatment, precipitation will occur if a trace of electrolyte is added. Dry albumin may be heated to 150° without becoming insoluble. Denaturation is therefore closely connected with the chemical nature of the protein, while agglutination is probably a result of the neutralization of the particle charge. In the presence of salts the coagulation is therefore dependent entirely upon the rate of denaturation. The kinetics of this reaction in the case of albumins and oxyhemoglobin have been studied by Lepeschkin,[102, 103, 104, 105] Chick and Martin,[35, 36, 37] Lewis[106] and others. Their results indicate that the phenomenon which occurs is closely related to hydrolysis. Lewis has shown further that the rate of reaction is at a minimum when the H^+ and OH^- concentrations are equal. The effect of the presence of salts is one of shifting the point of minimum rate.

Effect of heat upon the milk constituents. The effect of heat upon the individual milk constituents has been the subject of numerous investigations. In most cases, however, the effect of heating the purified substance has been studied. Data obtained in this way is not always of practical value in formulating a theory of the mechanism of the heat coagulation of milk since the behavior of the constituents of the product are markedly influenced by each other when found in unstable equilibria in the complex substance, milk.

Lactalbumin, though not studied so intensively as egg albumin, is quite similar to it with respect to its coagulation temperatures. They differ, however, in the type of coagulum formed by heat. Egg albumin congeals to form a solid mass while lactalbumin forms a flocculent precipitate. Orla-Jensen and Plattner[134] found that there was some coagulation of the albumin when milk was heated at 60° for 5 hours. Babcock[10] could find no change on heating milk at 65° for 20 minutes, while Freudenreich[58] reports that 15 to 20 per cent is coagulated on heating at 68° to 69.5° for 30 minutes. Rupp[163] found that no albumin was coagulated when milk was heated for 30 minutes at 62.8°, but at 65.6°, 5.75 per cent of the albumin was rendered insoluble; and at 68.3° and 71.1° for

30 minutes the amount increased to 12.75 per cent and 30.78 per cent respectively. These results indicate that in milk the rate of denaturation of lactalbumin at temperatures below 60° is very slow. The time/temperature coefficient has not been determined.

The effect of heat upon the calcium caseinate molecule is probably of a hydrolytic nature. Many references are to be found in the literature dealing with the cleavage of pure casein under conditions varying as to reaction and salt content of the medium and temperature of heating. However, results available to date are not of great assistance in gaining an understanding of the changes involved in the heat coagulation of milk, wherein the chief substance responsible for the change observed is calcium caseinate.

Svedberg, Carpenter and Carpenter [186] have observed that the molecular weight of pure casein is doubled when it is heated to 40°. These authors believe that the heating causes a polymerization or association of the molecules to form larger aggregates. The effect of higher temperatures of heating upon the particle size of the calcium caseinate of skim milk has been investigated by Nichols et al.[132] The weight-optical-particle-size distribution curves were determined for samples of the calcium caseinate in skim milk separated at 40° and preheated to 65° and 95°. The particle-size distribution of calcium caseinate was found not to be affected by this treatment.

Wright,[221] in a study of the racemization of natural and heated proteins, showed that within the limits of the temperatures studied (up to 120° for 30 minutes) heat does not affect the constitution of the casein molecule. In a later study [222] the decrease in solubility of the casein of milk powder was found to be a function of the time and temperature of heating, an increase in the heat applied causing a decrease in solubility.

The salt equilibrium of fresh milk is considerably altered by heat and the changes which heat cause in the milk salts undoubtedly play an important part in the heat stability problem.

Söldner [178] was the first to call attention to the fact that when milk is boiled a precipitation of a portion of the calcium phosphate occurs. The amount of fixation is proportional, in general, to the amount and duration of the heat applied.

Palmer [140] explains the partial fixation of the calcium of milk during heating on the basis of the precipitating effect of heat upon the colloidal $CaHPO_4$, the calcium phosphate natural to cow's milk.

Leighton and Mudge [100] have shown that an endothermic reaction accompanies the appearance of visible curd when milk is coagulated by heat and show that this same phenomenon takes place when artificially prepared milk serum is heated. They believe, therefore, that the heat absorption is incident to the precipitation of some of the calcium and magnesium salts normally present in milk. The thickening of sweetened condensed milk upon standing or subjection to high temperatures is attributed to this same reaction of the salts.

Leighton and Deysher [99] showed further that, when coagulation

occurs, there is a precipitation of the calcium and magnesium as phosphates and citrates with an absorption of heat. The greater the heat-resisting properties of the milk, the less is the amount and speed of formation of this precipitate.

Solutions of milk salts were prepared by Zoller [224] similar to those found in milk and quantitative and electrometric determinations were followed with temperature changes. Zoller found the "loss of calcium was progressive with the time and intensity of heat treatment. The H^+ concentration increased proportionately with removal of the buffer material by calcium. Doubling the quantity of citrates above normal, although not changing the initial pH of the solutions greatly reduced the precipitation of the calcium phosphate and at the same time maintained a higher final pH."

The citric acid content of normal milk which is about 0.20 per cent is destroyed by ashing of milk (p. 22). Lesser amounts of heat appear not to destroy this constituent. Splittgerber,[183] using solutions of mono-, di-, and tricalcium citrate, and of citric acid, heated them to dryness and continued the drying for five hours without observing a great loss in weight. Sommer and Hart [179] found the citric acid content of cow's milk to be unchanged even after heating to 118° for one hour.

The change occurring in lactose as a result of its subjection to high temperatures is not of great importance in the problem of heat coagulation except so far as the reaction of the medium is altered.

Milroy [126] showed that when milk was heated there was a slight increase in acidity. Whittier and Benton,[214] in a detailed study of the changes in hydrogen-ion concentration and acidity of milk during heating, found that the heating of skim milk at temperatures near the boiling point causes first a drop and then a rise in the titratable acidity of the milk. The hydrogen-ion concentration increases continuously. The initial drop in titratable acidity has been shown by previous investigators to be due to loss of carbon dioxide from the milk; the increases in titratable acidity and in hydrogen-ion concentration are due to the formation of acids from certain constituents of the milk. During coagulation the rate of change of hydrogen-ion concentration is considerably lessened due to buffer readjustments not yet explained in detail. At the same time there is an uneven distribution of the free acid between the whey and the curd, due probably to adsorption of the acid by the curd.

In later experiments these authors [215] showed that acid is formed during the heating at a rate which is a direct function of the time and of the lactose concentration, and concluded that lactose is the principal source of the acid produced by heating milk.

The brown color which forms in milk during heating was first shown by Orla-Jensen and Plattner [134] to be dependent upon the presence of both the lactose and casein. If either one of these constituents is heated separately under the same conditions of reaction and application of heat as is used in heating the milk, no color develops. More recent investiga-

tions have confirmed this finding, but the mechanism of the reaction is still unknown.[74, 153, 212, 221]

A study of the effect of heat upon the color of evaporated milk has shown that an increase in heat whether encountered during forewarming, sterilization, or storage, produces an increase in color.[209]

The heat coagulation of milk. The problem of heat coagulation in milk is in reality a problem of the heat stability of the calcium caseinate system. Little is known concerning the mechanism of its coagulation or concerning the equilibria values of the various phases in milk at the different temperatures. The research done upon this problem is fragmentary in the sense that each worker has, as a rule, considered only some single phenomenon. A complete theory for the processes incident to the coagulation of milk by heat is therefore lacking. About 12 hours' heating at 100° is necessary to coagulate fresh milk. At 130° coagulation occurs in approximately one hour, while at 150° the reaction occurs in approximately three minutes.[14] Considerable variation may be noted not only with different milks, but also between milks from different quarters of the udder of a single cow.[28] In view of the high temperatures necessary for a comparatively rapid coagulation of the caseinate system, it seems quite evident that albumin has little to do with its stability toward heat.

The velocity of coagulation is a function of the concentration as well as of the temperature and time of heating. As the concentration of solids-not-fat is increased the time and temperature of coagulation are decreased. The relationship between the temperature and the time of coagulation is of a logarithmic nature.[80, 211] The time of coagulation of an evaporated milk prepared from milk of good quality wherein the concentration of solids-not-fat is approximately twice as great as in normal milk, varies between 10 minutes at 131°, 60 minutes at 114.5°, up to 7500 minutes at 80°.

The time of coagulation at a definite temperature varies also with forewarming. In the case of milk containing a normal concentration of its constituents this treatment decreases its time of coagulation while in the case of those milks reduced to a concentration of 15 to 18 per cent or more solids-not-fat, the stabilities are markedly increased.[211] Hence, in the evaporated milk industry, the milk is raised to boiling temperature in a "hot well" prior to its processing.

The quantitative relationship between the temperature and time of forewarming has been studied by Leighton and Deysher,[99] and by Deysher, Webb and Holm,[49] who have found marked variations in stability with variations in forewarming temperatures. In addition, the time as well as the temperature must be considered. Rapid improvement in resistance to heat coagulation results from increases in temperature up to 90° to 100°. Above 95° the change is very small, but in some cases can be effected with increases in temperature up to 120° for 10 minute periods of forewarming. When time is chosen as the variable, improvement may be noted with increases in the time up to 30 minutes at a temperature of

95°. At higher temperatures the same improvement may be effected in shorter periods of time.

The presence of fat in relatively large aggregates may act as nuclei about which coagulation of the calcium caseinate system can proceed. When milk fat is present in an unhomogenized state in normal concentrations, the coagulation time and temperature are affected but slightly. However, when a product of higher fat content is homogenized the resulting fat clumps appear to act in a purely mechanical way as nuclei about which the casein may gather during heating. It follows, therefore, that those factors which affect the degree of fat clumping will also affect the heat stability of the product.

Doan and Minster,[50, 51] and Webb and Holm,[208, 210] have investigated the effect of preheating temperature and homogenization pressure upon the heat stability and fat clumping of milks and creams of different fat percentages. The effect of homogenization is small when low concentrations of fat and normal concentrations of solids-not-fat (9 per cent) are present. With increases in fat content or in homogenization pressure a marked lowering of stability is noted, other conditions being equal. Maximum heat stability results when the product is preheated and homogenized at 80°, while minimum stability is encountered at 60°. The general nature of these relationships are shown in Figure 29, compiled from the data of Webb and Holm. Rehomogenization, or the use of a second stage valve wherein the second pressure is lower than the first, breaks up the larger clumps and consequently increases heat stability over that encountered after only one homogenization.

The feathering of some homogenized creams when added to coffee may be due to the reduction of heat stability through the use of too high pressures, as shown by Webb and Holm.[208] That the salt content of the cream or of the coffee,—chiefly the presence of relatively large amounts of calcium,—may be an important factor has been shown by Tracy and Ruehe.[193]

The most important factor concerned in the heat stability of milk is to be found in the nature of the ionic equilibrium attained by its numerous salts and the manner in which the different ions affect the calcium caseinate system. Concerning the nature of these reactions little is as yet known.

Sommer and Hart[180] are of the opinion that there is a critical balance between the acidic and basic salt components which is necessary for maximum stability of milk to heat coagulation. They believe that in most cases coagulation is due to an excess of calcium and magnesium.

Rogers, Deysher and Evans[160] have shown, however, that the value of the salt balance as determined analytically in a normal milk is not an indication of the heat stability of the evaporated material made from the normal sample. The conclusion is that the salt balance of normal milk, while a factor, is not the controlling factor since milks of the same salt balance as determined analytically react differently toward heat.

The stabilizing effects of various buffer salts (phosphates, citrates,

carbonates, etc.) upon milks have been known for a long time, though the mechanism of their action is still not clear. Benton and Albery [22] showed clearly that variations in the concentrations of these ions, especially those of citrates, have a greater effect upon the coagulation temperature than has a slight variation in the hydrogen-ion concentration, excepting in cases

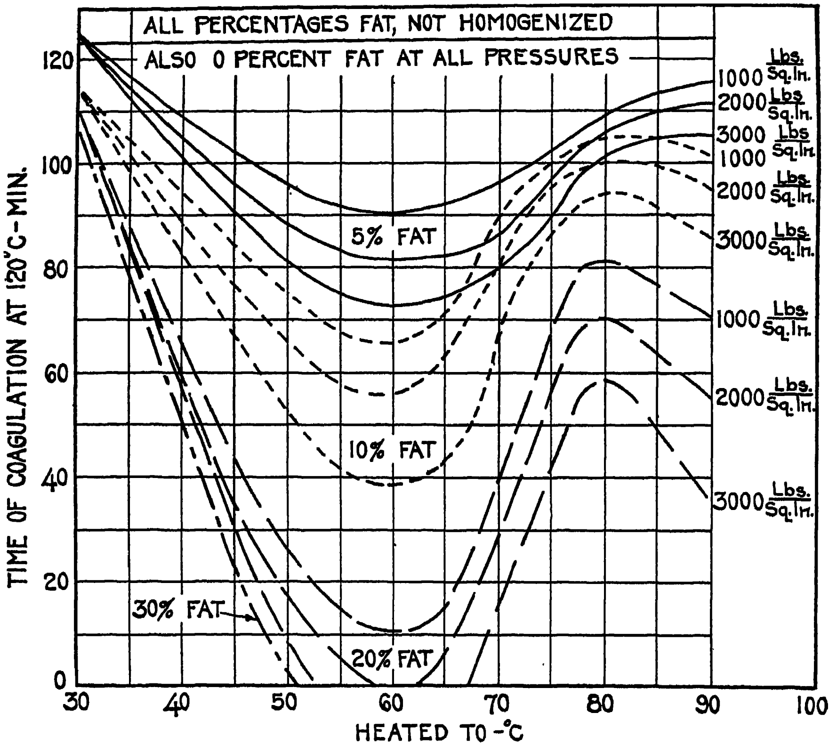

FIG. 29.—Variations of coagulation time of milk of normal solids-not-fat content with changes in forewarming temperature, fat content and homogenization pressures. No homogenization below 50°.

wherein the hydrogen-ion concentrations are of values above or below the range of pH 6.58 to 6.65. Both factors must be considered and as these authors have stated "the optimum combination of pH and salt balance is probably a resultant of several variables and consequently is an expression of the colloidal peculiarities of the particular samples. It is different in different milks."

An investigation of the effect of different salts upon the heat stability of milks has been conducted by Webb and Holm,[211, 212] and by Holm, Webb, and Deysher.[81] The following discussion is based upon their results.

No relationship was found between the heat stability and the composition and other properties of the milk from four cows during the course

of a lactation period. However, a detailed study of the effect upon stability of the addition of different salts to milk yielded some interesting results. Representative data plotted in Figure 30 show in general the relationships found.

The right half of Figure 30 represents the heat stability of milks to which active positive ions were added while the left side of the figure represents the stability of milks to which a salt containing a strong negative ion was added. The top curve represents the typical behavior of all skim milks of normal concentration while the lower curves show the

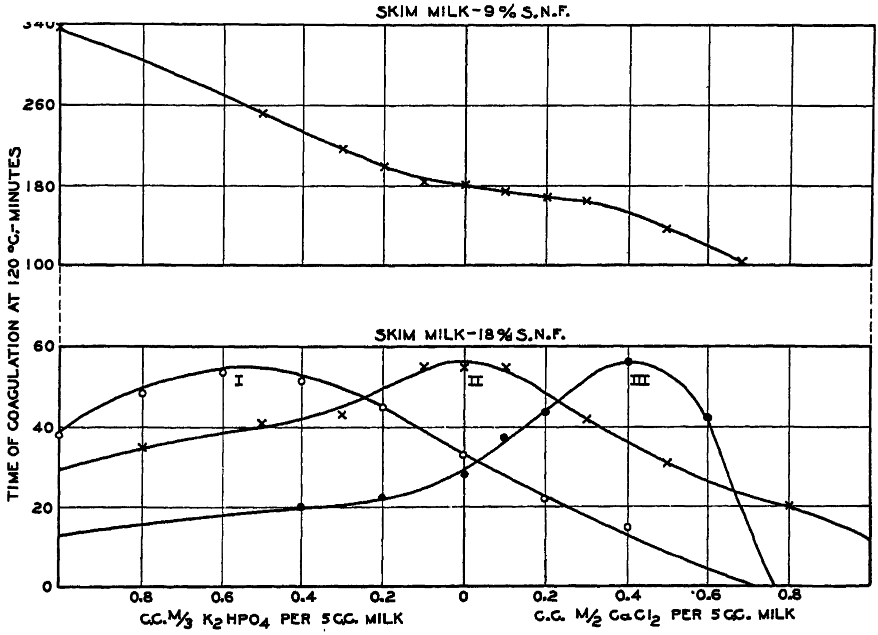

FIG. 30.—The effect of the addition of varying amounts of different electrolytes upon the heat stability of normal and evaporated samples of the three different types of milk.

behavior of different types of milk which may be encountered when the normal skim milks are condensed to half their original weight.

No method is at present known by which these three types of milk may be distinguished before evaporation. The alcohol test and the phosphate test [152] have been used to give an indication of the heat stability which may be expected in a milk after evaporation but these are not entirely satisfactory.

Of the three types of milk shown in Figure 30, I is the most common. It is increased in heat stability by small additions of phosphates or citrates. Milk II has attained maximum stability in its normal state, the addition of strong positive or negative ions causing a decrease in stability.

Additions of small quantities of $CaCl_2$ or of some other chloride,

including hydrochloric acid, cause a marked increase in the heat stability of milk III. This milk is comparatively rare, apparently being secreted by from 10 to 20 per cent of the cows of a normal herd. The scarcity of milk III may perhaps be traced to a seemingly unstable salt equilibrium. If a type III raw milk is placed in storage there will be a gradual shift in the heat stability curve of its evaporated product through type II to type I, although little change will be noted in the stability curve of the unevaporated sample. The shift from type III to type I will be accelerated by the development of lactic acid during storage, but it may also proceed without the accompaniment of a measurable change in hydrogen-ion concentration. This indicates a shift in the original calcium phosphate-calcium caseinate equilibrium during storage.

There is no clear division among the three types of milk, since they merge into one another and numerous curves can be obtained showing a difference in degree of variation.

Many different salts may be used with substantially the same results as are obtained with $CaCl_2$ and K_2HPO_4. Differences in valency of the ions concerned generally account for the differences in ionic concentration found necessary to produce a given result. The chlorides of H, Na, K, Ca, Mg, Ba, Al and Th furnish a source of strong cations while the sodium and potassium citrates and phosphates may be used when strong anions are required.

The action of calcium is more drastic in nature than that of phosphate. The addition of small quantities of phosphate to a milk as a neutralizer to increase heat stability is useful if a phosphate stabilized milk is encountered (curve I), while if the milk is stabilized by calcium (curve III), the addition of phosphate does not increase stability but at the same time there is no marked lowering of stability. This fact accounts for the success of phosphate salts as neutralizers in evaporated milk manufacture.

Unpublished results of Holm, Webb and Deysher show that the addition of salts to milk before forewarming and condensation shift the normal course of the heat stability curve of evaporated milk. If a small quantity of calcium chloride is added to a fresh milk which normally has a stability curve of the type of milk II, Figure 30, the resulting curve is shifted toward curve I. If a phosphate salt is added to the fresh milk the heat stability curve is shifted toward curve III. This behavior is to be expected since it is the normal adjustment arising from an excess of either a strongly positive, or negative, ion, for this particular milk.

When milk is coagulated by heat the actual process of thickening occurs in a relatively short period of time just prior to the separation of the curd, or coagulation. During the thickening there is a release of calcium and phosphorus into the serum; and Chorower [38] believes that, as the calcium is released from the casein molecule, the molecule increases its hydration and swells, thus increasing the per cent volume of the suspended phase and causing thickening. The increase in body may also be due to increase of particle sizes by aggregation to the point where plasticity effects are introduced.

The relation of forewarming to the thickening of sweetened condensed milk during storage was studied by Leighton and Mudge[100] who found that use of temperatures of forewarming near the boiling point resulted in a product which jelled during storage, while the use of lower temperatures did not produce this effect. Later experiments by Greenbank, Steinbarger, Deysher and Holm[67] show that temperatures of forewarming which produce a sweetened condensed product that will thicken also seem to increase the hydration capacity of the proteins which is probably a cause for the gelation effect. This is in agreement with the idea of Chorower.[38]

The work detailed indicates that in milk the mechanism of the coagulation process is dependent upon a number of interdependent reactions, the qualitative and quantitative natures of which are still unknown.

Alcohol Coagulation

Investigators were of the opinion for some time that the ease with which a milk could be coagulated by alcohol furnished direct evidence of its instability toward heat. The coagulation of milk by alcohol involves somewhat different processes and can not always be depended upon to indicate susceptibility to coagulation by heat. Alcohol has a dehydrating action and denatures proteins quite readily. The casein is precipitated as calcium caseinate and there is no sudden release of salts as is the case in heat coagulation. There is, however, a decided change in both titratable acidity and pH. High temperatures have marked effects upon solubilities and rates of reaction. One could hardly expect a test carried out at room temperature to parallel exactly the behavior of a complex mixture like milk at temperatures above the boiling point. Nevertheless, as Dahlberg and Garner[48] pointed out, the alcohol test is more useful than an acidity determination in predicting the stability of milk destined to be evaporated and sterilized by heat. In general milks which are not precipitated by 70 per cent alcohol are sufficiently stable for this purpose, though occasionally a milk stable to heat will give a positive reaction.

It should be pointed out here that slow additions of alcohol allow for denaturation and hence, for most accurate results, additions should be made rapidly and the time factor kept small.

As milk gradually sours it becomes susceptible to alcohol precipitation shortly before it loses its stability to sterilizing temperatures, though the precise pH at which the change takes place is not the same in all milks. As instability thus induced is constantly encountered in the industry the usefulness of the test is obvious. Milks are not infrequently encountered, however, which though otherwise of good quality are alcohol positive when secreted. Colostrum milk is always alcohol positive. Milk secreted at the end of lactation or when the mammary tissue is slightly irritated or inflamed though suitable for food is usually unstable to heat and, as a rule, is alcohol positive.

Sommer and Binney[181] have shown that alcohol coagulation, like heat

coagulation, is affected by the salt balance, and that the addition of small amounts of Ca or Mg may render a milk alcohol positive while citrates or phosphates oppose this effect.

Benton and Albery [22] in studying the effect of certain anions found that the "±" point (+ to 75 per cent alcohol, — to 70 per cent alcohol) indicated the optimum concentration beyond which further addition of the salt in question reversed the effect.

It should perhaps be mentioned that the alcohol test was originally suggested as a measure of the freshness and purity of market milk, especially when combined with a color indicator for acidity (Morres' Alizarol test). Its unsuitability for this purpose was well shown by Ayers [9] who also gives a careful review of previous literature on the subject. He also brings out another point of similarity between alcohol and heat coagulation, namely,—that milk rendered alcohol positive by acidity or rennet treatment can be stabilized by forewarming to 90° or higher.

Rennet Coagulation *

One of the most interesting and important properties of milk is that of clotting or setting into a stiff gel on addition of one of the several representatives of the enzyme called chymosin or chymase. This clotting is commonly referred to as a coagulation but, as a matter of fact, it is only the smooth, gel-like curd which possesses properties essential for the production of the principal kinds of cheese, especially those which require ripening. Any condition of the milk employed for the manufacture of such cheese which causes the curd to assume the properties of an ordinary coagulation instead of the desired jelly-like clot is to be considered an abnormality. Faulty technic in the initial stages of manufacture may also cause the clot to be faulty in this respect.

Source and distribution of chymase-like enzymes. The ability to coagulate milk is apparently possessed by all peptic proteases and, to some extent, by tryptic and ereptic † proteases. This fact accounts for the apparently widespread distribution of chymases in the digestive tract of all mammals, birds, reptiles, most fish, many of the lower forms of animal life as well as in extracts from leaves, fruits and seeds of various plants. Certain fungi and bacteria also possess milk-clotting enzymes, distinct from the acid-producing enzymes which may indirectly cause the coagulation. Some of the so-called phytochymases may be employed for cheese making, but in most cases their optimum action is exhibited under conditions which are not favorable for true chymase.

The milk-clotting power of all preparations of plant and animal origin other than those from the stomach of suckling mammals is attributed by Oppenheimer [138] to the particular protease or proteases present and not to the presence of a specific chymase. So far as the phytochymases are concerned their proteoclastic action is not deep-seated.[47] Both Effront [53] and Oppenheimer [138] (p. 1108) review the literature on the occurrence of

* Revised to March 1, 1934.
† It is doubtful whether a pure erepsin has ever been tested.

phytochymases. The milk-curdling enzyme in the berries of *Solanum elaeagnifolium* has been purified and studied in some detail by Bodansky.[26, 27]

The only true chymase is obtained from the stomach of suckling mammals. This enzyme is more commonly called rennase or rennin, and an extract containing rennin, as well as dried preparations from the same, is called rennet. The commercial rennet sold in clarified saline solution or in powdered form is usually obtained from the inner lining of the so-called fourth stomach (abomasum) of young calves and lambs.

Rennin versus pepsin. It is probable that commercial rennet usually, if not always, contains a certain amount of pepsin. The debate on the question whether rennin and pepsin are identical or distinct enzymes is one of long standing. An excellent review of all phases of the literature on this controversy which appeared during the first quarter of the present century is given by Oppenheimer[183] (p. 978). The burden of proof has rested upon those who held the view that the enzymes are distinct, inasmuch as almost all investigators have noted that the purest preparations of pepsin * still possess milk-clotting ability. Investigators therefore sought to concentrate the chymase activity without causing a corresponding concentration of peptic activity. Many investigators have succeeded in accomplishing this, thus pointing to the non-identity of true chymosin and pepsin. Fenger[55] nearly accomplished the purification of rennin possessing no peptic activity. Tauber and Kleiner[187] seem to have actually done so, although the complete absence of peptic activity of their preparation is questioned by Holter[82] on the grounds that the methods employed by Tauber and Kleiner for measuring peptic action are not sufficiently sensitive for estimating slight activity. Although the point involved is one of great importance for understanding the biochemistry of milk clotting by rennin it is not essential for deciding the question of the specificity of rennin and pepsin; the final proof of their specificity has now been furnished by Tauber and Kleiner[188] through the demonstration that crystalline pepsin in proper dilution completely destroys extremely active, highly purified rennin, presumably by digestion. Trypsin likewise accomplishes this, but not erepsin.

The milk-curdling ability of stomach extracts from mature animals has long been attributed to an enzyme called parachymosin. Oppenheimer believes that this is none other than pepsin itself, which seems probable. However, his view that rennin is normally transformed into pepsin is rendered untenable by the finding already mentioned,—that rennin is digested by pepsin.

True rennin has been regarded since Hammarsten's[70] earliest studies as arising from an inactive zymogen (pro-rennin) which is activated by acid. (See Oppenheimer,[122] p. 998, for review and also Tauber and Kleiner[189] and Kleiner and Tauber[90] for recent work.)

Potency of rennin. True rennin is an extremely powerful biocatalyst. No method has yet been devised for measuring its primary

* This is likewise true of crystalline pepsin.[187]

action, the exact nature of which is still an unsettled question. Holter [82] has suggested a possible means for its quantitative definition. The most commonly employed criterion of rennin action is the amount of natural or reconstituted milk or casein solution which will be coagulated by a unit quantity of rennin in some arbitrary period of time under arbitrarily selected conditions with respect to temperature, pH, added calcium salts, etc. Some of the recently devised viscosity methods are obviously dependent upon coagulation.

The coagulation phenomenon, however, is secondary to the actual changes produced by the enzyme. The justification for employing this secondary phase of the process for measuring rennin activity rests upon the belief that, "at constant temperature the time required for coagulation is inversely proportional to the quantity of rennet used." This is the so-called law of Segelcke and Storch (1870). No set of data with varying quantities of rennet exhibits exact agreement of $t \times m = k$ (where $t =$ time required for coagulation and $m =$ amount of rennet). The relationships hold fairly well within a certain range of "*t*" or "*m*." Grimmer and Krüger [68] found that "*k*" increases to a maximum with increase in "*m*" and that the following logarithmic relationship holds between the different "*m*," "*t*" and "*k*" values

$$\log\ (k - t_1) - \log\ (k - t_2) = c(m_2 - m_1)$$

when $k =$ maximum value of k, and t_1, t_2, m_1 and $m_2 =$ corresponding values of t and m, and $c =$ a factor determined for the individual cases based on the amount of rennet used.

According to Lenk [101] the original law holds true only when "*t*" falls between 6 and 14 seconds. Lüers and Diem [110] as well as Christen and Virasoro [39] found, in conformity with Grimmer and Krüger, that "*k*" *decreases* with decreasing quantities of rennet when milk is employed but, when a calcium-caseinate-calcium-phosphate complex is used, "*k*" *increases* with decreasing quantities of rennin. Holter [82] finds that both natural and synthetic milks show decrease of "*k*" with decrease of "*m*" when the test is carried out at 25°, instead of at 35° to 40°, indicating a destruction of rennin at the higher temperatures when added to synthetic milk, which does not occur in natural milk. Furthermore, he finds that, if the results obtained at the lower temperature are recalculated on the assumption that the coagulation time represents the sum of the primary and secondary phases of the process, it is possible to obtain two values of "*k*" (one of which he calls "*x*") both of which are constant, at least through the range of enzyme concentration which he employed. The new "*k*" and the "*x*" are calculated by assuming that each value of $k = m\ (t - x)$, where $x =$ time required for the other of the two phases of the process, and solving for x when several of these equations are available. Holter admits that his results may be interpreted either as representing the two major phases of the coagulation process or that they may be due to the possibility of a certain latent period being required before the normal enzymatic reaction occurs.

Because of the inadequacies of the Segelcke-Storch law and the importance of the other factors influencing the coagulation phase of the phenomenon a comparison of the maximum activity values obtained for various purified rennins is of doubtful significance. It is possible to state only the activity found under the conditions employed. Fenger [55] obtained a rennin by a precipitation procedure, one part of which coagulated 3,900,000 parts of commercial pasteurized milk in 5.75 min. after slight acidification with lactic acid (pH probably about 6.5). One part of the dry purified rennin obtained by Lüers and Bader [111] through repeated adsorption on kaolin at pH 5.1 and elution with phosphate buffer at pH 6.97, coagulated 5,000,000 parts of slightly acid milk (pH probably about 6.2) at 35° in 12.2 min. These investigators gave their product a calculated activity of 16,440,000 by converting the activity found to a 40 min. coagulation time, using the Segelcke-Storch law. The purified rennin of Tauber and Kleiner [187] obtained by fractional isoelectric precipitation in 50 per cent ethanol at pH 5.4, had a maximum milk clotting power of 4,425,000 units. This represents the amount of a reconstituted skim milk preparation at pH 6.2, containing added $CaCl_2$, which is clotted in 10 min. at 40° by 1 part of dry rennin. The activity was doubled by increasing the acidity of the substrate solution to pH 5.4.

Nature of rennin. Fenger [55] describes rennin as an acid albumin which readily dialyses through parchment. The purest preparation obtained by Lüers and Bader [111] was not a protein but apparently contained a small amount of protein as impurity, judging from the extremely weak biuret and xanthoproteic reactions, negative ninhydrin and Millon's reactions, and a nitrogen content of only 0.687 per cent. On the other hand the purified rennin obtained by Tauber and Kleiner [187, 189] is described as a thioproteose, irreversibly inactivated by alkali, very easily soluble in dilute acid, not coagulated by heat, and diffusible through membranes on long dialysis; it gives a slightly positive xanthoproteic test, a pink biuret test, but negative Millon's and Hopkins-Cole tests; it is soluble in water at its isoelectric point, pH 5.4, but precipitates in 50 per cent ethanol at this pH; it is slowly inactivated by ethanol. Elementary analysis of this rennin shows 14.40 per cent N, 1.19 S and no P. The enzyme, if present in this preparation adsorbed on protein, exhibits no marked tendency to be transferred to edestin.[188]

The marked difference in nitrogen content of the preparations having nearly equal milk coagulating power obtained by Lüers and Bader and by Tauber and Kleiner, respectively, remains to be explained.

Constituents of milk affected by rennin. There is no conclusive proof that rennin exerts an effect on any constituent of milk other than the casein. It has been demonstrated beyond doubt that cations are essential for the clotting process, the calcium of milk acting chiefly in this capacity. The extreme view of Briot [31] is that rennin acts only on the calcium phosphates of milk, but this view has no foundation.

There is normally a definite interval between the formation of the altered casein and its coagulation, and this can be prevented by addition of

$CaCl_2$ at the end of the first stage of the reaction. Loevenhart [108] concluded from this observation that rennin also performs the function of making calcium ions available for the coagulation stage of the process. This view has been accepted by some writers, but the evidence on which it is based is circumstantial and should therefore be interpreted with caution. The only calcium salts known to be present in milk in a form such that the calcium is not readily available for the rennin coagulation are colloidal calcium phosphates. If rennin is able to liberate calcium ions from colloidal calcium phosphate, it should be able to do so outside of milk as well as in milk. Rennin produces no change whatever when added to a colloidal calcium phosphate sol protected by gelatin.

Stages in the clotting process. The coagulation or clotting of milk by rennin is commonly regarded as due primarily to a direct effect of the enzyme on the casein of the milk, which probably exists, at least in cow's milk, chiefly as calcium caseinate or (and) as calcium phospho-caseinates, a chemical complex of calcium caseinate and calcium phosphates. The casein which has been altered by rennin is called paracasein.* Since the pioneer experiments of Hammarsten [71] however, it has been rather generally accepted that there are two independent stages in the clotting process. The first stage is the rennin action itself, the second is the precipitation of the altered casein.

Rennet clotting as an indirect effect of rennin. There have been some writers who have held that rennin causes clotting indirectly through its destructive effect on stabilizing substances in the milk. The hypothesis of Schryver [175] is that the colloidal casein is stabilized by substances adsorbed from its surroundings. It is assumed that milk contains casein-coagulating substances, e.g., $CaCl_2$, but that these can not act upon the casein until the rennin clears away the protectors from the surface of the casein particles. This hypothesis was incapable of complete proof chiefly because it was found that casein which has been precipitated from milk by $CaCl_2$ retains all its original properties when redispersed; whereas casein precipitated by rennin can not be precipitated again by the enzyme.

Alexander's view,[1] although expressed earlier, is more specific. The hypothesis is that the rennin destroys a protective effect which lactalbumin exerts on the colloidal casein. This hypothesis was based on his ultra-microscopic studies [2, 3] on milk and also on the belief that the casein in milk is an irreversible colloid (suspensoid) whereas lactalbumin is a reversible colloid (emulsoid). Inasmuch as emulsoids will protect or stabilize suspensoids, it was not a far step to conclude that lactalbumin stabilizes the casein solution and that the precipitation of the latter by rennin must be due to a destruction of the protective qualities of lactalbumin.

In support of this theory it was shown that lactalbumin will stabilize colloidal suspensions of silver chloride and calcium phosphate, but fails to do so after peptic digestion. It is also stated that protective colloids such

* The terms caseinogen and casein are also employed synonymously with the terms casein and paracasein, respectively. The term paracasein seems to have been employed first by Hammarsten.[72]

as gelatin and gum arabic are capable of preventing the coagulation of casein by rennin.

These views have been widely quoted, but are subject to severe criticism. Palmer and Richardson [141] conclusively demonstrated that rennin exerts no effect on lactalbumin and also that protective colloids like gelatin and gum arabic have no detrimental effect on rennet clotting. In fact they usually favor it, both as to the rate of clotting and greater firmness of clot. Fuld [62] earlier found that there is no difference in rate of coagulation with rennet of milk diluted with milk serum and of milk diluted with the same serum previously digested with rennet. This serum was prepared by neutralizing the filtrate obtained after acid precipitation.

Somewhat different theories regarding the destruction of protective colloids by rennin have been advanced by Marui [115] and by Linderstrøm-Lang.[107] The former believes that the colloidal "Grundlage" of milk which coagulates when rennin is added, consists of colloidal calcium phosphate and large micellae of calcium caseinate. In normal milk these are protected by a coating of much more finely dispersed casein. The action of the rennin is upon this protective colloid, changing it to paracasein, a non-protective colloid, with the resulting destruction of the main colloidal system.

Linderstrøm-Lang's theory, which is supported by Holter,[82] is based on the hypothesis which has developed in Sørensen's laboratory, that the natural casein of milk is a protein component system, consisting of at least three substances, differing in solubility, calcium-binding capacity and phosphorus content. The colloidal stability of this system, as it exists in milk, is regarded as due to the protective action of one of its components. Rennin attacks this component with the resulting destruction of the entire casein system. Cherbuliez and Meyer,[34] who have separated four components from the casein system, find that only one of them is markedly affected by rennet, thus lending some support to Linderstrøm-Lang's theory. However, their finding that this component may vary from 5 per cent to 61 per cent of the casein system renders the theory untenable unless it should eventually be shown that variations in the behavior of milk towards rennet are explained by the wide variations in that proportion of the casein system primarily affected by rennin.

Fundamental questions involved in rennin action. If it be accepted that casein is the only constituent of milk affected by rennin, it is of great importance to know the changes involved. The theory of the clotting phenomenon hinges very largely on this point. There are several fundamental questions involved: First, what is the chemical and physical character of casein in milk; second, are the changes produced by rennin chemical or physical, or both; third, how does rennin produce these changes?

Chemical and physical character of casein in milk. It has been known since the early observations of Schübler [176] that casein is merely suspended in cow's milk. The colloidal nature of this suspension has been abundantly verified by dialysis and ultrafiltration methods. The brilliant

appearance of the caseinate particles in very active Brownian motion has been described by Alexander and Bullowa [1] and by Bleyer and Seidl.[25] In fact, milk furnishes one of the most beautiful laboratory demonstrations of colloidal dispersion. Kreidl and Neumann [97] obtained photo-ultramicrographs of two stages in the rennet coagulation phenomenon. This evidence, together with the fact that electrolytes, e.g., $NaCl$, $CaCl_2$, $AlCl_3$, will precipitate the particles in the order of efficiency corresponding to the increased valence of the cations leads to the conclusion that the caseinate particles are true suspensoids (hydrophobes) carrying a negative charge. Colloids of this class, however, are not dispersed by the cations which precipitate them as is the case with casein which is readily dispersed by Na or Ca ions to form stable sodium or calcium caseinate solutions. The latter are identical with milk when viewed by the ultramicroscope.

On the other hand the caseinate dispersion of milk is not a true emulsoid (hydrophil) like gelatin and albumin which are more or less insensitive to cations. This caseinate, therefore, appears to form dispersions intermediate between suspensoids and emulsoids, dispersing in water like the latter yet retaining its visibility in the ultramicroscope and its sensitivity toward electrolytes.

Hammarsten,[70] from the first, regarded the casein in milk as a calcium caseinate-calcium phosphate complex, in fact as "calcium phosphocaseinate." The latter name, although euphonious, has the disadvantage that it implies the existence of a true double salt, which Hammarsten did not have in mind and which the late Dr. Porcher and his school of thought, who employ the term extensively, likewise have been careful to deny. As a matter of fact, the compound which is called calcium caseinate is most probably a true calcium phosphocaseinate if, as seems likely, the second and third hydrogens of the orthophosphoric acid esterified with certain of the amino acids in the casein molecule react with calcium. The correct conception of the term "calcium phosphocaseinate," as it is now commonly employed, is that of a colloidal calcium phosphate (or phosphates) sol protected by a calcium caseinate (or caseinates) sol in a manner as yet imperfectly understood. (A hydrotrophic effect is not excluded.) The "complex" is made by dispersing casein in lime water and bringing the pH back to that of milk with H_3PO_4 solution. The colloids are separable by mechanical means, as Van Slyke and Bosworth [201] showed, although their complete separation is not possible even with the supercentrifuge, according to Porcher [148] (p. 82). These facts increase the difficulty of understanding the nature of the colloidal complex.

The presence of the two colloidal systems, one protecting the other, plays an important role in the rennin-clotting phenomenon, which will be pointed out later.

Nature of changes produced by rennin. Some investigators have concluded that rennin affects only the physical state of the calcium caseinates while others have maintained that the change in the casein is strictly chemical. Some of the physical theories will be mentioned first.

Loevenhart's [108] idea was that rennin causes an association of casein

particles into paracasein, a less highly dispersed colloid. This theory has been revived by Beau,[20] who explains the association as a physico-chemical polymerization of the colloidal "calcium phosphocaseinate," analogous to the polymerization of formaldehyde to produce plastics. There is also a strictly chemical aspect to Beau's theory, namely, that a certain number of the free carboxyl and amino groups in the casein molecule are united by secondary valencies, which are opened by the action of the rennin. These are then reunited through calcium and phosphate radicals, providing physical aggregates which become larger and larger, through polymerization, until the clot is formed.

Laqueur,[98] however, regarded paracasein as a more highly dispersed colloid than casein.

According to Inichoff [85] rennin peptizes casein, aided by hydrogen ions and bivalent metallic cations. This view is apparently supported by the statement of Bleyer and Seidl [25] that calcium paracaseinate particles are always smaller than calcium caseinate particles, and their Brownian motion twice as rapid. An especially interesting idea expressed by Mellanby [120] is that rennin converts an emulsoid, casein, into a suspensoid, paracasein. Inasmuch as casein already partakes of the nature of both classes of colloids, as previously pointed out, the rennin effect from this point of view must be one of degree only.

There have also been hypotheses relative to the formation of adsorption compounds of casein and other substances. One such hypothesis advanced earlier by Mellanby [119] is that paracasein is an adsorption complex of casein and rennin, the casein requiring a definite quantity of adsorbed rennin before it will coagulate as paracasein, the amount being inversely proportional to the calcium ions in the milk. Bang [18] advanced another, somewhat different, hypothesis, namely that rennin merely changes the adsorption affinity of casein for calcium, casein being the first member of a series of compounds with increasing affinity for the base, those of greater affinity being paracaseins. This view is based on a belief, apparently supported by experimental evidence, that the various paracaseins can be made to clot again with rennin. The same reclotting phenomenon had been previously observed by Peters [144] but this had, however, already been explained by Hammarsten [71] as due to NaCl in the rennet extract, paracasein being much more sensitive than casein to sodium ions. Inasmuch as this explanation has been verified by Laqueur [98] it can be accepted with certainty that casein once coagulated by rennin has lost its sensitivity toward the enzyme.

Turning now to the chemical theories of rennin action, mention should first be made of Hammarsten's classic theory. He believed that rennin hydrolyzes the calcium caseinate-calcium phosphate complex into a calcium paracaseinate relatively poorer in calcium phosphate and a relatively calcium-rich whey proteose. In spite of the fact that this theory appears to be supported by much experimental work,[7, 8, 68, 75, 130, 157, 174, 178, 182] careful study by Bosworth [28, 29] and Gaeke [64] has failed to show that

casein and paracasein have a different chemical composition. The elementary composition and Hausmann numbers are given in Table LXXXV.

Table LXXXV.—Identity of casein and paracasein as shown by elementary composition, nitrogen distribution, and rate of racemization in alkali.

Elementary composition					Hausmann numbers			Specific rotation in alkali		
	Casein (Bosworth)	Casein (Gaeke)	Para-casein (Bosworth)	Para-casein (Gaeke)		(Gaeke) Casein Total N	Para-casein Total N	Time	(Wright) Casein	Para-casein
	per cent	per cent	per cent	per cent		per cent	per cent	hr.	$[\alpha]_D$	$[\alpha]_D$
Carbon	53.50	53.20	53.50	53.05	Melanin	1.53	1.66	0.5	102.5	103.5
Hydrogen	7.13	7.09	7.26	7.03	NH_3	10.23	10.31	2.5	89.5	88.5
Nitrogen	15.80	15.63	15.80	15.63	Mono-amino	65.31	63.90	9.0	74.5	74.5
Phosphorus	0.71	0.73	0.71	0.809	Di-amino	22.94	24.05	50.0	55.0	55.0
Sulfur	0.72	1.015	0.72	1.009		....	....	169.0	39.0	38.0

In spite of the relatively constant, although small, fraction of the casein molecule which most investigators find is hydrolyzed as a proteose-like substance (Porcher found this to be about 4 per cent of the original casein nitrogen) during the conversion of casein to paracasein, it is evident that this does not produce a significant change in the composition of the original casein. It has never been determined whether this splitting is essential for the production of paracasein or whether it is merely the result of true peptic properties of the rennin or of contamination of the rennin with pepsin. As the differences between casein and paracasein have come to light it has become increasingly difficult to believe that these are explained by this small loss of nitrogen. This problem is again raised by the claims of Tauber and Kleiner [187] that pure rennin has no peptic action.

Petry [145] found that rennet lost its power to produce whey proteose from casein, but not its ability to coagulate milk, when digested in Na_2CO_3 solution at 37°. Bosworth found no constant relation between the amount of whey proteose produced from calcium caseinate and the amount of casein or rennet used. Holter,[82] who questions the complete absence of peptic power in pure rennin, nevertheless regards it as having no significance in the milk clotting phenomenon. Some consideration may also be given the claims of Cherbuliez and Meyer [84] that one component of the casein system in milk, in fact that comprising the smallest proportion of the total (about 2 to 5 per cent), is not coagulated by rennet. However, the split product which has been found in whey does not appear to be a primary protein. A solution of this question awaits a test of the Tauber-Kleiner rennin on a synthetic milk made from that component of the casein system which is coagulated by rennin.

Chemical identity of casein and paracasein is indicated also by an identical rate of decline in the specific rotation of the proteins in two per cent solutions of 0.5 N NaOH as found by Wright,[221] and by a study of

their formol titration which was made by Inichoff.[85] The racemization data are given in Table LXXXV.

It is evident that chemical differences between casein and paracasein are not to be sought on these grounds. Bang[18] was apparently the first to suggest a difference between casein and paracasein based on their affinity for calcium. He regarded this as only a temporary difference depending on conditions brought about in the presence of rennin. Palmer and Richardson[141] studied the base-combining capacity of casein and paracasein

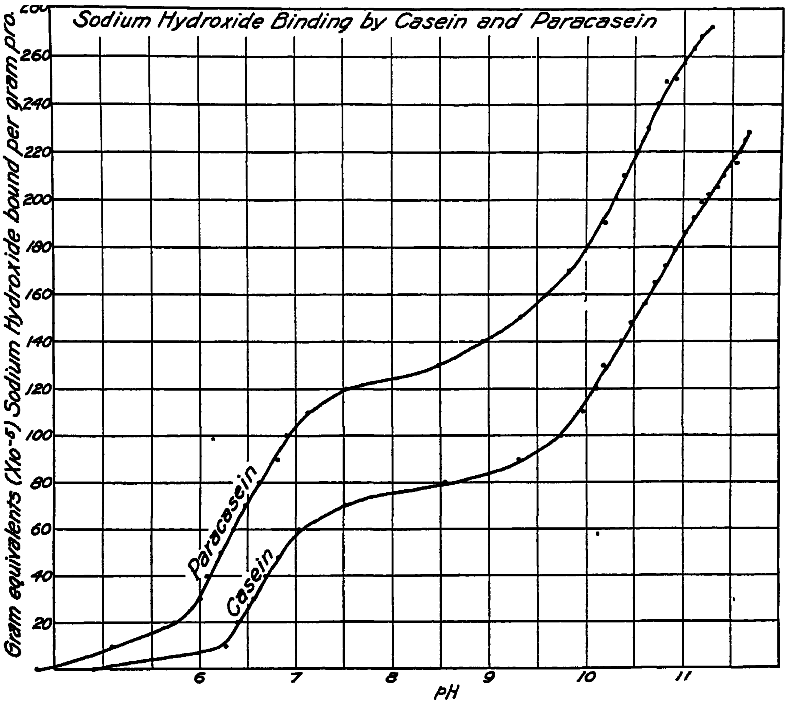

FIG. 31.—Sodium hydroxide binding by casein and paracasein.

using a strictly comparative method throughout the entire range of hydrogen-ion concentration which might exist in milk. The results, shown graphically in Figures 31 and 32, indicate clearly that capacity to combine with base is one of the most important chemical differences between casein and paracasein concerned with the rennet coagulation phenomenon. It appears, moreover, that neither casein nor paracasein dissociates as a polyvalent acid, which is contrary to the conclusion of Van Slyke and Bosworth[201] and of Cohn.[41] The so-called acid compounds that have been postulated by the former occur on the first buffer slope of the curves; therefore, they are not to be regarded as definite entities. Only one calcium caseinate and one corresponding paracaseinate is indicated, the former containing 80 to 90 $\times$ 10^{-5} and the latter 150 $\times$ 10^{-5} gram equiva-

lent of base. It is only at the pH of fresh milk that there is a quantitative ratio of 1:2 between the calcium bound by casein and paracasein, although at this pH the definite compounds are still in the process of formation.

The outstanding chemical differences between casein and paracasein are in their solubility, maximum acid- and base-binding capacity, and

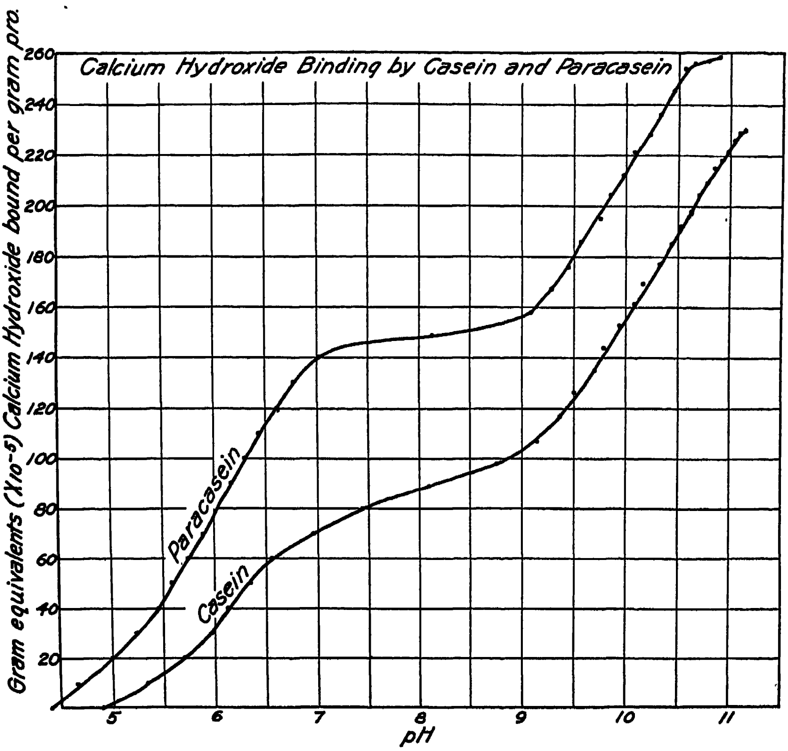

FIG. 32.—Calcium hydroxide binding by casein and paracasein.

titration curve with acid and base. The first two properties have been established by Pertzoff.[142, 148]

The following quotation from Palmer and Richardson summarizes their conclusions regarding the significance of their results:

"It is obvious that rennin, acting on an incompletely-formed calcium caseinate in colloidal dispersion at the pH of milk, converts it into a much less completely formed calcium paracaseinate, the chemical binding capacity of which for both base and acid is permanently altered. The nature of the molecular rearrangement or surface change (since a substance in colloidal dispersion is altered) causing the increase in binding is not clear, but it is certainly not explained on the basis of simple molecular division or peptization. Neither of these changes could alone increase the gram equivalents of alkali and acid bound per gram of protein. It is obvious that the instability of the highly unsaturated (with respect to base) paracaseinate, augmented by the higher temperatures employed in rennin clotting, is responsible for the greater sensitivity toward cations, and explains its coagulation."

According to Nicholas,[181] it is possible to distinguish casein from paracasein biologically by means of the different rates at which they produce a precipitin reaction with sera from animals sensitized to the respective proteins.

Chemistry of the clotting process. As already pointed out, Hammarsten first demonstrated that rennin action in itself does not bring about coagulation, this being dependent on the presence of calcium. This has been substantiated by a number of investigators. Practical cases in which calcium deficiency interfered with cheesemaking have been cited by van Dam [196] and by Koestler.[92, 93]

Investigators have not agreed, however, as to the role of the calcium. Ringer [157] believes that the calcium salts of milk combine with the paracasein to form the curd. Arthus and Pagès [7] believe that paracasein unites with calcium ions. Fuld [63] regards the coagulation as a special case of mutual suspension and precipitation of colloidal substances, with special reference, however, to a mixture of casein and paracasein. Bang [18] regards the coagulation stage as a mere salting out of calcium-rich paracasein by means of free calcium salts. Van Dam [196] holds that it is the colloidal calcium together with the calcium already united with casein which causes the precipitation of paracasein and that the soluble calcium salts of milk play no part in the coagulation except indirectly through helping to control the pH. Van Slyke and Bosworth [201] are of the opinion that calcium paracaseinates, being insoluble in the presence of calcium salts, precipitate as soon as the rennin acts. According to Milroy,[126] calcium ions, at least as $CaCl_2$, increase the hydrogen-ion concentration and also the activity of the rennin. Cosmovici [44] holds a similar view, namely, that the effect of calcium ions is added to that of hydrogen ions. In addition he finds that the consistency of the curd depends on calcium ions.

These confusing and contradicting views make it obvious that the role of calcium can not be explained on simple chemical grounds. The colloid chemistry of calcium caseinate and calcium paracaseinate solutions shows that the clotting of these sols is identical in principle with the gelation of inorganic suspensoids by means of ions, in this case, cations. At the present time these phenomena are perhaps best explained by the theory that the suspensoid sols are stabilized by an electrical double layer (the so-called Helmholtz double layer) around each colloid particle. In the case of negatively charged sols, in which class calcium caseinate and calcium paracaseinate evidently fall, the outer layer consists of hydrogen ions. If these are replaced by a sufficient number of positively charged ions of higher charge, e.g., calcium ions carrying two positive charges, the colloid particle will readily precipitate, gelation being determined by the rate of replacement and precipitation. In the case of calcium paracaseinate, with its greater calcium combining capacity, it appears that the farther removed the paracasein is from saturation with base, the fewer the calcium ions that will be required to replace the outer layer of hydrogen ions and cause precipitation. The base-binding curves in Figures 31 and 32 show that the calcium paracaseinate formed in milk by rennin is so far removed

from saturation that one should expect that monovalent cations, e.g., Na or K, will cause precipitation. This is the case, as Hammarsten first showed in explaining the apparent anomaly that calcium paracaseinate will clot again with rennet extracts. Hammarsten found that the large amount of NaCl in these extracts is sufficient to cause the reclotting. The effect of other cations will be discussed later.

Beau [21] employs the findings regarding the greater acid- and base-binding capacity of paracasein in comparison with casein, to support his theory, mentioned above, that rennin opens secondary valency unions between —COOH and —NH_2 groups in the casein molecule. Experimental evidence is still lacking that casein combines with alkalis or acids through secondary valences, either normally or after rennin has acted on it. Miyamoto and Schmidt [127] have obtained evidence that casein forms complex ions with calcium, but were unable to show by the methods employed by them that secondary valence plays a part in the formation of such ions. In their experiments the phosphoric acid in the casein molecule was responsible for about 10 per cent of complex ions forming in a calcium caseinate solution.

Richardson and Palmer [141] have obtained experimental evidence that the electrokinetic explanation of the part played by calcium ions and other cations in the clotting phenomenon may be extended to include the action of rennin itself. They found indications that the electrokinetic isoelectric point of rennin is in the neighborhood pH 6.9 to 7.0, below which it is positively charged. (Tauber and Kleiner [187] found the precipitating isoelectric point of rennin to be pH 5.4.) Inasmuch as rennin lowered the electrophoretic velocity of calcium caseinate and calcium phosphocaseinate micellae when the sols and the catalyst solution possessed opposite electric charge, but not when their electric charge was alike (above the electrokinetic isoelectric point of rennin), it could be postulated that rennin exerts its effect as a coagulating agent, in part at least, by sensitizing the colloidal micellae towards the free cations of milk through a preliminary reduction in the electric charge. Further proof of this hypothesis was obtained in the finding that calcium paracaseinate micellae are not thus affected by rennin, which is in line with the fact that casein once coagulated by rennin is no longer sensitive to the enzyme.

Mlle. Brigando [80] rejects the possibility of an electrokinetic effect of rennin on the caseinate micellae on the grounds that there could not be enough molecules of rennin to produce such an effect. However, if one employs Wiegner's [216] maximum estimate of 3×10^{12} micellae per c.c.[3] Tauber and Kleiner's [187] estimate of rennin potency and an assumed molecular weight for rennin of 6,000 (as a proteose) it is possible to calculate from these figures and the Avogadro constant for colloidal particles that there will be 7,000 to 8,000 molecules of rennin available for each micella calculated to be present.

The calcium salts of milk, however, may play another role in the clotting process. Most investigators, apparently, have given little attention to the nature of the coagulum in their experiments with rennin. Ham-

marsten [72] held that calcium paracaseinate gives only a flaky precipitate with $CaCl_2$ or NaCl, whereas the calcium paracaseinate-calcium phosphate complex, which he regarded as a natural constituent of rennin-treated milk, gives a clot. Hammarsten [71] had earlier observed that calcium phosphate *per se* is not essential in the coagulation phenomenon when employing casein sols, the phosphoric acid being replaceable by H_2SO_4 and H_2CO_3 and the Ca by Ba, Sr or Mg. Lundberg [112] soon found that HCl, HNO_3 and oxalic acid could also replace H_3PO_4. Marui [115] has shown that the colloidal calcium phosphate may be replaced by any hydrophobic colloid which is both sensitive to calcium ions and capable of being protected by calcium caseinate. For example he was able to employ a mastic sol as well as calcium oxalate. Porcher [149] employed arsenic and silicic acid sols.

In all of the foregoing studies the second colloid presumably has been present as a member of the so-called complex with calcium caseinate. That this is not essential seems to be indicated by the observation of Palmer and Richardson [141] that mixtures of gelatin and calcium paracaseinate give only a flaky precipitate with NaCl, whereas mixtures of calcium paracaseinate and a colloidal solution of calcium phosphate (in gelatin) give clots.

It would seem, therefore, that the principal role of the colloidal calcium phosphate, either as a member of the calcium phosphocaseinate complex or as a separate colloid, is to provide an increased concentration of micellae so that the sol will set into a gel rather than flocculate. However, Richardson and Palmer [156] have secured rennin gelations of calcium caseinates containing 3 to 4 per cent casein, so that the colloidal calcium phosphate is apparently not necessary under all conditions. Porcher [147] maintained that the coagulation of calcium caseinate alone is not possible. It is not entirely clear whether the colloidal phosphate acts more efficiently in the role under discussion when present in the complex rather than as a separate colloid, but it seems reasonable to assume that it might do so when under the colloidal protection of its semi-hydrophilic colloidal protector.

Porcher [147] has shown that increasing amounts of calcium phosphate in the complex sol not only increase the firmness of the clot, but also the rapidity of coagulation. If this result is solely the effect of increasing the concentration of colloidal micellae it should occur also with calcium caseinate sols alone. This has not been tested.

Physico-chemical changes during clotting. Milk shows very little change in physical properties other than consistency during the two stages of the rennin clotting phenomenon. Porcher [148] (p. 431) found that the viscosity of calcium caseinate solutions at pH 7 or lower decreases when treated with rennet under conditions which do not cause coagulation, but that it is unaffected at higher pH. The proteolytic action of the rennet is believed to be the cause of this effect. A rise in temperature of 0.149° during 10 minutes of activity, also a rise in freezing point of 0.018 to 0.019° was observed by Fuld,[62] when comparing unclotted and clotted milk. A very slight increase in conductivity during the clotting stage was

noted by Inichoff,[85] amounting to 0.41×10^{-4} mhos, but Reichel and Spiro[154] found no change. Neither Inichoff[85] nor Milroy[126] found a change in hydrogen-ion concentration at any stage in the process. This has been regarded as proof that no calcium is actually fixed by the para-casein during clotting. Cosmovici[48] observed an increase in surface tension amounting to above five per cent as the result of clotting.

Factors influencing rennin action. The wide difference of opinion held regarding the number and character of the changes produced when rennin acts upon casein, and the lack of unanimity as to which changes are the more important precludes any extensive discussion of the factors which influence rennin action. Furthermore, no methods have been developed by which the primary and secondary phases of the clotting phenomenon may be satisfactorily distinguished. The method suggested by Holter[82] for studying the rennet coagulation law and the electrokinetic attack suggested by the work of Richardson and Palmer[156] seem promising.

The general problem has three major aspects: (a) the factors which affect the rennin itself; (b) the factors which affect the specific substrate upon which rennin acts, at least so far as concerns its chemical and physical properties which are modified by the enzyme; and (c) the factors which normally affect the relations between any enzyme and its substrate.

With respect to the enzyme itself an important factor which must be considered in any experiment is the instability of rennin, which is sensitive to many factors, especially temperature and hydrogen-ion concentration. Rennin being an enzyme of mammalian origin undoubtedly has an optimum temperature in the neighborhood of body warmth, but actual data on this point are confused by the fact that the optimum temperature for clotting has been used as the measure of this property of rennin. Unfortunately rennin is rather unstable at the usual temperature for optimum clotting, i.e., 40°. Rennin also undoubtedly exerts its optimum effect at some definite pH or within some pH range, which is possibly the pH range of greatest stability in solution, i.e., 5.3 to 6.3 (see Holwerda[83]). Some influence on rennin action could be expected to result from contamination with pepsin or trypsin which, as noted previously, destroy rennin. Not a great deal is known regarding the effects of antiseptics on rennin but the enzyme is surprisingly stable towards formalin, as shown by Porcher[148] (p. 328). A concentration of formaldehyde of 1:850 has very little retarding influence even after contact for over one hour. Another factor which may, at times, require consideration is the presence of natural paralyzers or anti-rennins. Oppenheimer[133] (p. 1003) reviews the question in some detail. If rennin is a positively charged colloid it would not be expected that cations could affect its activity but that anions should do so. Neutral salts such as NaCl seem to be without effect on rennin, but it has been claimed both that calcium salts directly favor the rennin action and also that they hinder it. Hirudin, which inhibits blood coagulation, and cephalin, which accelerates blood coagulation, have no effect on rennin action. Very active heparin, another blood anticoagulant,

may retard rennin action by reacting with the rennin (Stone and Alsberg[185]).

With regard to the substrate upon which the enzyme exerts its action it must be remembered that one is dealing with an apparently specific substance in a distinctly characteristic colloidal dispersion, at least so far as natural milk is concerned. However, the latter does not seem to be a necessary part of the rennin action inasmuch as caseinates of the alkali metals are also changed to paracaseinates, as was shown by Van Slyke and Bosworth.[202] It seems probable, however, that definite changes in the caseinate molecule itself or its colloidal micellae could be brought about which would modify the rennin action. It is probable that cations play their role here rather than on the enzyme itself. Antiseptics such as formaldehyde, which react with protein, might be expected to hinder the reaction but Trillat[194] and later Porcher[148] (p. 326) showed that formalin (40 per cent formaldehyde) in concentrations up to 1:2000 have only a mild effect on the rate of coagulation, with normal clotting; larger amounts have greater effects on the rate and also affect the character of the clot. Bearn and Cramer[19] were able to retard rennin action by adding heated (60°) rennin to milk along with active rennin. The result was explained on the grounds that the heated rennin formed a "zymoid" (a term coined by Bayliss[18]), or intermediary compound, with the casein without exerting the usual effect of rennin and thus delayed the action of the active rennin. This theory has a renewed interest in connection with the view of Moir[129] that the delayed coagulation of pasteurized milk with rennin results from a coating of the casein by denatured but incompletely coagulated lactalbumin. This may be looked upon as a type of "zymoid" formation.

As to the general relationships between rennin and its substrate the more important factors would be the absolute and also the relative concentration of the two components.

Factors influencing the coagulation. The rate of coagulation and the character of the coagulum are influenced by several important factors, e.g., temperature employed for coagulation, previous temperature of the milk, hydrogen-ion concentration, concentration of casein and calcium in the milk, character of cations used for coagulation, and a number of other factors whose relation to coagulation is more easily understood. It will be sufficient to discuss only the less apparent factors.

The optimum temperature for coagulation, that is, the temperature at which coagulation is the most rapid with given quantities of milk and rennin, apparently coincides with the optimum temperature for rennin action. For calf rennin, at least, this is 40° to 42°. Below and above this temperature the rate of coagulation decreases so that at 10° to 15° and 60° to 65° no coagulation occurs. The clot, which is firm at the optimum temperature, becomes softer at the lower temperature and tough and stringy at the higher. Within normal clotting range, the elasticity of the curd has been shown by Allemann and Schmid[5] to be directly proportional to the temperature increase with no apparent maximum.

The fundamental factors determining temperature effects are not as clear as they might be. The rate at which paracasein becomes available for precipitation is obviously one factor. Another is the degree of instability of the colloidal calcium caseinate. The base-combining curves in Figures 31 and 32 indicate that the paracaseinate formed at the pH of fresh milk is considerably removed from a stable compound. If, as has been stated by Bang,[18] the calcium-binding capacity of paracasein increases with rise in temperature, one may expect a corresponding increase in instability of the paracaseinate formed in milk. A third factor is the rapidity of neutralization of the electrical charges of the paracaseinate particles. The concentration of the cations obviously determines in part this phase of the reaction. With a given amount of available cations, the relation of temperature to ionization also explains, in part at least, the increased coagulability with rise in temperature.

The firmness of the clot is influenced by a number of factors including the time of setting, amount of rennin, reaction of milk, temperature of clotting and other factors. All these have been studied quantitatively by Allemann and Schmid.[5] Cosmovici[44, 45] showed that the retractability of the clot and the expulsion of whey depend upon a proper hydrogen-ion concentration. Hill[78] called attention to the fact that the firmness of the clot with rennet, or curd tension, is a characteristic of the milk of individual cows. He devised a method for measuring the curd tension quantitatively, analogous to that employed by Allemann and Schmid. The variations in curd tension of milk from different cows has been shown by Weisberg, Johnson and McCollum[218] to be due primarily to variations in the concentration of the suspensoid phase, i.e., the colloidal caseinates and phosphates, with the amount of fat a minor factor.

If milk is boiled before being treated with rennin, the rate of coagulation is retarded greatly, and there is also a very detrimental effect on the character of the clot. Similar, although much less pronounced effects usually follow pasteurization.

The opinion commonly held as to the cause of these results, namely, that heat precipitates available calcium salts, rests on circumstantial evidence of a very deceiving nature. This evidence is two-fold: (1) that heating precipitates calcium phosphate from milk, (2) that the addition of soluble calcium salts restores the original properties of the milk. Some new facts having an important bearing on this evidence have been obtained by Mattick and Hallett.[118] They held samples of milk for 30 minutes at various temperatures between 105° and 209° F. (41° and 98° C.) and compared the total and diffusible phosphorus and calcium with that in unheated milk. No significant effects on phosphorus were noted up to 178° F. (81° C.). Above this about 3.5 per cent of the diffusible phosphorus was made nondiffusible. Higher temperatures produced no greater effects. More marked effects were noted on the calcium. At 135° to 140° F. (57° to 60° C.) about 0.6 per cent of the diffusible calcium was made nondiffusible. This rose to 2.0 per cent at 145° to 150° F. (63° to 66° C.), and above this temperature the results varied

between 2.5 and 3.6 per cent, indicating that the effect of heat reaches a maximum, as in the case of phosphorus, but at a lower temperature. If diffusible calcium also represents ionized calcium, it is difficult to reconcile these relatively small changes with the general effects of heat on milk that have been frequently alleged to be caused by effects of heat on the calcium salts of milk.

It was first observed by Söldner[178] that milk whose coagulability with rennet had been retarded by heating showed a gradual and further decrease in coagulability the longer it was kept after the heat treatment before applying the rennet test. This was confirmed by Stassano and Talarico[184] and by Rupp[168] who observed also that milk which had been heated to 55° to 65°, coagulated with rennin more rapidly than the raw milk. Price,[150] also, has noted an accelerating effect on rennet coagulation of heating milk to 54° to 68°. These observations have been extended still further by Mattick and Hallett,[118] but the cause of these hysteresis-like effects has not been discovered. Moir[129] believes they are due to the gradual accumulation of denatured, but not completely coagulated, lact-albumin on the caseinate micellae.

Palmer[140] suggests that previous temperature effects should be sought on the ground of the effect of heat on calcium caseinate. This idea is substantiated by the experiments of Michaelis and Marui[124] who found that the coagulation-retarding effect of previous high temperatures can be duplicated by subjecting casein solutions to various temperatures previous to treatment with $CaCl_2$ and rennin. Porcher[148] (p. 371), however, has regarded the entire phenomena as due to the effect of heat on the colloidal calcium phosphate portion of the calcium phosphocaseinate complex. In support of this it is shown that aliquots of a neutral (pH 7.03) calcium caseinate heated for 30 minutes at 40°, 65°, 80° and 100°, respectively, and then converted by lime water and H_3PO_4 into the complex at pH 6.78 show no difference in time or character of rennet coagulation whereas aliquots of a complex (pH 6.69), similarly treated, show the usual retarding effects of the increasingly severe heat treatments. Moreover, it is shown that such a complex whose coagulability had been entirely destroyed by autoclaving at 125°, could be restored to nearly normal by "recharging" it with calcium phosphate, i.e., addition of lime water and H_3PO_4. These experiments seem convincing, but would be more so had the recharging of the heated sols with calcium phosphate been accomplished by other means than addition of alkali and acid. The probable effect of these treatments on the casein itself cannot be denied until disproved.

Electrokinetic evidence of heat effects on both calcium caseinate and calcium phosphocaseinate complex sols was obtained by Richardson and Palmer.[156] They found that heat increases the cataphoretic velocity of casein solutions, which means that it increases the electric charge on the micellae. Although rennin was able to reduce this charge, as shown in these studies, the reduction was not sufficient to lower the velocity to that found in control unheated solutions. This result offers the first logi-

cal colloidal explanation of why it is necessary to add highly active cations ($CaCl_2$) to heated milk in order to cause heated milk to clot normally with rennin. These results throw no light on the hysteresis-like effects of heat noted by Mattick and Hallett, because the casein solutions employed were heated only at one temperature (that of boiling water) for 1 hour, and sufficient time elapsed before the heated sols were studied in the cataphoretic tube to have secured the maximum effects.

In contrast to the evidence for an increased potential of casein as the result of heating, Ballowitz [11] has obtained indirect evidence for a theory advanced by Michaelis and Marui,[124] but which Marui [115] was later unable to substantiate, that the effects of heating milk on its subsequent coagulation by rennet are due to the adsorption of essential calcium from the milk by the casein. This should reduce the potential of an electronegative colloid. He finds that after boiling milk with $CaCl_2$ a smaller proportion of the added Ca but all of the added Cl is recovered in the ultrafiltrate. Also, according to his results, milk loses about 10 per cent of its ultrafiltrable Ca on boiling and the addition of this quantity of Ca as $CaCl_2$ restores in part the original coagulability with rennet. Analogous results are reported by Ballowitz [12] using a casein solution in sodium acetate-phosphate buffer. Final judgment on these results must be reserved until it can be determined to what extent they are attributable to the reaction of the added $CaCl_2$ with the phosphate systems, both soluble and colloidal, in milk and the relation to them of the marked fall in pH which accompanied the alleged adsorption of Ca by the casein.

It has been appreciated since the early work of Courant [46] that the reaction, i.e., the acidity, of milk is of great importance in determining the rate of coagulation and the character of the curd. Van Dam [106] first obtained data indicating a relationship between rapidity of coagulation and hydrogen-ion concentration. Coagulation of paracasein at the pH of fresh milk is, however, not a question of difference in isoelectric point, because casein and paracasein have the same isoelectric point. Allemann's [4] work indicated that the optimum hydrogen-ion concentration for most rapid coagulation with rennin is at pH 5.35. Michaelis and Mendelssohn [121] showed, however, that in the presence of calcium ions there is a definite optimum zone lying between pH 5.99 and 6.40 which therefore coincides with the zone of complete conversion of casein to paracasein. In this work hydrogen-ion concentrations corresponding to pH 5.5 and 7.0 were clearly outside the zone of coagulation.

The base-binding curves of casein and paracasein (Figs. 31 and 32) show that calcium paracaseinate is relatively much nearer its isoelectric point than calcium caseinate at 6.0 to 6.4. As a matter of fact, it may be best to consider that the zone of maximum coagulability is the isoelectric zone of calcium paracaseinate in the presence of calcium ions. This view is in harmony with the theory of the coagulation stage of the rennin phenomenon put forth in a preceding paragraph, and is supported by the experiments of Michaelis [125] and associates [122, 123] showing that cations,

especially bivalent cations, displace the isoelectric point of electro-negative ampholytes toward neutrality.

It has been known since the early observations of Eugling[54] and Söldner[178] that the addition of oxalates to milk is unfavorable to and may prevent entirely rennet coagulation. Inasmuch as ammonium, sodium and potassium oxalates and oxalic acid itself each exert a somewhat different effect, the results obtained by various investigators have led to some confusion. The problem has been studied by Arthus and Pagès,[7] van Dam,[190] Milroy,[126] Platon,[146] Porcher[148] (p. 394) and others. The differences observed are to be explained on the grounds that in some cases the effect of the oxalate may be primarily on the Ca ions, in others it may reduce the Ca ions and also increase the pH above 7.0 and in still others there may be destruction of the colloidal calcium phosphate as well as a double decomposition of the alkali oxalate and calcium caseinate with the resulting replacement of part of the calcium caseinate by a caseinate of the alkali metal. Sodium fluoride and sodium citrate additions to milk also exert an unfavorable effect on coagulation with rennet.

Casein and calcium and hydrogen ions being so closely involved in the coagulation process, it is to be expected that the concentration of casein and calcium exerts a marked effect both on the rate of coagulation and the character of the clot. If milk is diluted with sufficient water, coagulation is both delayed and incomplete, the clot being soft and fragile. The partial restoration of original properties secured by the addition of $CaCl_2$ suggests that it is the dilution of calcium ions which is the more important factor. However, it must always be remembered that the addition of $CaCl_2$ increases the hydrogen-ion as well as the calcium-ion concentration of milk. The concentration of casein must be of some significance, at least for determining the character of the clot. The entire question, however, requires further investigation.

The functions of calcium ions in the coagulation process have been given considerable attention in these paragraphs. While the matter is of little practical importance, it is of great theoretical interest to know that any metallic cation can replace calcium in the performance of its functions. Even such rare elements as cerium, lanthanum, neodymium, praseodymium, samarium, and thorium have been shown by Fronin and Mercier[60] to hasten the coagulation of milk. Loevenhart[108] studied the effect of both cations and anions on the coagulation of paracasein. In his study none of the monovalent cations except lithium caused coagulation, although this is contrary to what has already been pointed out for sodium. The divalent cations arranged themselves in the following Hofmeister series in the decreasing order of their effectiveness:

$$Mn^{++} > Ni^{++} > Co^{++} > Fe^{++} > Ca^{++} > Ba^{++} > Sr^{++} > Mg^{++} > Be^{++}$$

The anions were without effect when comparing chlorides, sulfates and nitrates.

Table LXXXVI.—Relative effectiveness of cations in accelerating rennin coagulation.

Concentration of salt	Order of effectiveness of chlorides of the elements								
Mg. mols per liter									
1	Ca 133	Zn 128	Ba 122	Sr 114	Li 106	Mg 66			
2	Ca 145	Zn 137	Ba = 133	Sr 133	Li 106	Mg 71			
10	Ca 295	Ba 250	Sr = 227	Al 227	Zn 149	Li 103	Mg 100	K 94	Na 87
20	Ca 526	Ba 400	Mg 270	Sr 266	Zn 175	Li 103	K 94	Na 81	
100	Ba 526	Mg 475	Ca 345	Sr 179	Li 89	K 71	Na 70	Zn 37	

Lörcher[109] studied the effectiveness of various cations for rennin coagulation of milk. A summary of some of his results is given in Table LXXXVI. The data must be interpreted in the light of the fact that they represent the cumulative effect of the added cations and the calcium ions already in the milk. The figures under the symbol of each element represent the relative coagulation time. Normal milk is represented by 100. A figure of 50 indicates double the normal time and a figure of 200 one-half the time.

It is interesting to note that at equimolar concentrations the various cations studied arrange themselves in a different series as the concentration increases. Ca and Zn rise to a maximum and then fall. Mg, Ba, and Sr show a continued rise up to the maximum studied, this being especially marked for Mg and Ba. The monovalent ions, Li, Na, and K show no rise with increasing concentration, Li being without any appreciable effect except to depress the rate of coagulation at the highest concentration, and the alkali metals being depressants throughout.

Ballowitz[11] has studied the relative effects of Ca, Ba and Mg to restore the rennet coagulability of heated milk. The rise in effectiveness of added Ca followed by a fall has been confirmed by several investigators, including Rüdiger and Wurster,[162] who have followed this with changes in pH. The observations of Lörcher on the other cations have not been repeated with the view of correlating them with effects on hydrogen-ion concentration. No study has been made of the relative effectiveness of the various cations in producing a firm clot at the same molar concentration. Differences are readily observed when the respective cations are added to portions of oxalated milk which are then coagulated by rennet.

Cheese Manufacture

Unripened cheese consists of the casein, most of the fat, insoluble salts and colloidal material, together with part of the moisture of the original milk in which are contained lactose, albumin, soluble salts, and other minor milk constituents. For the purpose of reducing the essential solids of milk to a concentrated form, the milk is coagulated either by the bacterial development of acid or by the use of rennet. The moisture is separated from the curd more or less completely by simple mechanical division, by the development of acid, by stirring, and by a moderate or high degree of cooking and pressing. The cheese is placed in molds, salted, and stored in curing rooms to ripen at definite controlled temperatures. Thus, by various mechanical means and by the use of specific agents, milk may be transformed into a less perishable and more highly concentrated product which may vary in type according to the method of manufacture and the specific agents used.

Classification of cheese. For purposes of discussion cheese may be classified as follows:

- Soft
  - Unripened
    - Cottage
    - Cream
    - Neufchatel
  - Ripened
    - Ripened by molds
      - Camembert
      - Brie
    - Ripened by bacteria
      - Limburger
      - Liederkranz
- Hard
  - Semi-hard
    - Ripened by molds
      - Gorgonzola
      - Roquefort
      - Stilton
    - Ripened by bacteria
      - Brick
      - Münster
  - Very hard
    - Without gas holes
      - Cheddar
      - Edam
      - Gouda
    - With gas holes
      - Emmenthal
      - Swiss
      - Parmesan

Rennet and acid curds. The coagulum produced by rennet has the property of elasticity to a marked degree and, under certain conditions, the ability to shrink, thereby causing the elimination of whey. Curd obtained by the action of acid alone is not elastic, but is open and sticky, and contracts to a less degree than that obtained by the rennet coagulation. The rennet coagulum carries down with it most of the insoluble salts of the milk but, in the case of an acid curd, the insoluble salts are rendered soluble by the acid and are lost in the whey to a comparatively great extent.[128, 178] Since the calcium becomes soluble more rapidly than the phosphorus and other ash constituents, the Ca/P ratio and also the percentage of calcium in the ash is comparatively low in cheese made under conditions of high acidity, particularly if the acid curd is washed. Rennet and acid types of cottage cheese do not show significant differences in calcium content.[113] The reaction of the rennet curd being close to neutral, conditions are more favorable for the development of numerous groups

of organisms than in the acid curd where the high hydrogen-ion concentration restricts such growth. Consequently, with the rennet curd manipulations and the use of special curing temperatures and ripening agents, it is possible to manufacture cheese with a great range of properties and variation in composition.

Hard and soft cheese. The moisture content of hard cheese varies from 30 to 40 per cent and under favorable storage conditions this type may be kept for a year or even longer. Soft cheeses contain from 40 to 75 per cent moisture and are more quickly perishable. In the manufacture of the hard cheeses the curd is cut finer, scalded at a higher temperature, and molded usually at a greater pressure than is the case in the manufacture of the soft varieties. The lactose disappears from the cheese in the course of a few days after making, particularly in the cases where the curd is scalded at a high temperature. However, lactose does not entirely disappear from the soft cheeses till they have been kept for one or two weeks. In a ripened hard cheese about one-third of the casein has been converted into a soluble form. Considerable quantities of amino acids are present, but only a small amount of ammonia. Cheddar and similar types of cheese retain about 80 per cent, and soft cheeses about 20 per cent of the milk calcium; the phosphorus content varies with the amount of protein in the cheese, approximately 38 per cent of the milk phosphorus being retained in soft and Cheddar type cheese.[118] A considerable amount of the casein of soft cheese is converted into a soluble form on curing, and most varieties have a high percentage of ammonia. The interior of certain hard cheeses is entirely anaerobic.[94] Ripening occurs uniformly throughout a hard cheese, but in soft cheese ripening proceeds from the surface toward the center of the mass.

Temperature of coagulation. The temperatures for coagulation may vary from 20° to 48°, but the working range for most kinds of cheese lies between 28° and 35°. Fleischmann has determined the relative efficiency of the coagulation process at various temperatures between 20° and 50°. He expressed his results as the relative quantities of coagulum formed at the different temperatures, the maximum being taken as 100 at 41°. His values are given in Table LXXXVII.

As a rule the soft cheese requires a low and the hard cheese a high coagulating temperature. Marked difference in the texture of the curd may be brought about by the use of different coagulating temperatures. Neufchatel and Cream cheese "set" at from 21° to 25° have a soft, pliable, jelly-like curd; Cheddar curd formed at 30° is decidedly firmer; and Limburger curd "set" at 33° to 34° is distinctly tough and rubbery. Allemann and Schmid [5] found that the elasticity of the coagulum increased in direct proportion to the temperature up to 41°.

Time of coagulation. The first visible signs of coagulation may occur in 5 to 8 minutes after the addition of rennet, although the time is usually longer. The beginning of coagulation is indicated when a permanent depression can be made by holding a thermometer horizontally on the milk. Just before coagulation begins, bubbles caused by moving the

Table LXXXVII.—Relative efficiency of coagulation at various temperatures.

Temperature	Relative efficiency	Temperature	Relative efficiency
° C.		° C.	
20	18	40	98
25	44	41	100
30	71	42	98
31	74	43	96
32	77	44	93
33	80	45	89
34	83	46	84
35	86	47	78
36	89	48	70
37	92	49	60
38	94	50	50
39	96		

finger rapidly through the milk tend to remain upon the surface while one can count ten; before this time they disappear quickly.

The combined action of the rennet and acid affect the character of the resulting curd and the manner in which the whey is expelled. Failure of one or the other to function normally may delay drainage and result in a defective cheese. The curdling period for hard cheeses is usually from 25 to 45 minutes; the softer cheeses like Camembert and Roquefort require from one to two hours. When the curdling extends over a long period, droplets of moisture collect upon the surface of the curd, increase in size, unite, and form a thin sheet upon the coagulum. Eventually the solidifying action of the rennet practically ceases. In most cases the rennet enzyme, though digestive in character, exerts only a negligible influence at the end of this period. Hard-curd milk is said [79] to produce better quality Cheddar cheese than milk in which the coagulated curd is soft. In cheese made from soft-curd milk the body is weak and mealy, and the fat loss in the whey is high. The yield of cheese is also low in the case of soft-curd milk.

Cutting of curd. The purpose of cutting is to allow part of the whey to escape from the curd. At the proper time for this operation, the curd has usually drawn away slightly from the sides of the vat and moisture has collected upon the mass due to the contraction and hardening brought about by the rennet.

That the moisture content of the curd is influenced by the size of the particles has been shown by Sammis and his associates.[165] Different vats of experimental Cheddar curd were cut into cubes ¼, ⅜, ½, and ¾ inches on edge. After 2½ hours the curd contained 50, 53, 58, and 70 per cent of moisture respectively. This variation in moisture was due only to the difference in the size of the cubes in the four vats.

In the case of Emmenthal cheese, it has been shown by Koestler [96] that large particles contain a greater percentage of moisture than fine particles, yet fine cutting causes a retention of moisture in the cheese by diminishing the flow of whey between the particles, resulting in slow and

often insufficient drainage and excessive acidity. This effect of fine harping of curd as a cause of moisture retention has been demonstrated by other workers.[117] Koestler showed that so-called cheese powder causes the retention of whey and may lead to a defective fermentation, often localized in the form of blow-holes or gassy areas.

The curd particles may vary in size from that of a hempseed to that of a cherry, and in same cases to that of an apple, depending on the type of cheese being made. In the case of certain kinds of soft cheese, the time at which the coagulum can best be cut is determined by the manner and extent of the gathering of the whey film on the surface. With hard cheeses the time for cutting is often indicated when the curd gives a clean split before the finger, with porcelain-like surfaces and no adhering particles, or when the curd breaks away from the vat with a sharp edge upon the application of pressure. Cutting the curd prematurely or by the use of a curd breaker instead of knives increases the fat losses in the whey.

In the preparation of certain kinds of soft cheeses the curd is not cut except for the breaking caused in transferring it by ladle or dipper into the forms. In making Cream or Neufchatel cheese the coagulum is poured directly upon the drain cloths without preliminary breaking or cutting. In most cases the curd is reduced gradually to pieces of a size which will cause the development of the desirable properties of the type of cheese being manufactured. Uniformity in size of the pieces is one aim of all cutting operations. Whenever the pieces are not reduced to a uniform size, part of them will be firm and elastic and others will be soft and full of whey. This is likely to result in a low-grade cheese.

A thin continuous coating spreads quickly over the entire surface of each freshly-cut curd particle. This elastic film is what retains the fat and, to a certain extent, the whey within the curd. Whenever these curd particles are broken, some of the fat globules are lost in the whey. A certain loss of fat in the whey is unavoidable. For Cheddar cheese this should not be more than about 0.3 per cent. If the curd particles are further divided by means of a harp, as in the case of Swiss cheese, the fat losses may reach 0.6 to even 1.0 per cent. In such cases the whey may be separated and the cream used for the manufacture of butter.

The inner portions of the curd particles are softer than the outer due to large amounts of whey present. It is important that the film on the outside of the curd particles does not harden too rapidly and thus prevent the escape of whey to the desired extent. Subsequent operations have for their purpose the hardening and contraction of the pieces of curd.

Acidity and hydrogen-ion concentration. The coagulating power of rennet varies with the acidity of the milk. The acidity influences the solubility of the calcium salts and the precipitability of the casein in an even more direct manner, and the acidity of the curd influences the texture and the subsequent ripening of the cheese. The correlation of acidity with the properties of the milk and curd has been made in the past with the acidity expressed in terms of titratability. Recently, however, satisfactory methods for following changes in hydrogen-ion concentration in

cheese have been perfected.[177, 207, 219] Electrometric measurements are made on either curd or whey by means of a potentiometer and quinhydrone electrode. It is well known that all milks are not buffered alike and that the acids present are only partially dissociated, and for these reasons titration figures do not give as true an indication of effective acidity as is given by a measurement of hydrogen-ion concentration, which may be expressed as a pH value.

The initial acidity of the milk as well as the subsequent formation of acid in the whey is a matter of major importance in cheese making. Certain kinds of cheese require comparatively fresh unripened milk, as in the cases of Limburger, Brick, Bel Paese, and Brie, while the milk for Cheddar, Camembert, and Roquefort must be of a higher initial acidity. Acidity as a result and accompaniment of bacterial activity is essential to the proper ripening of nearly all kinds of cheese. In this connection it is interesting that 95 per cent of all bacteria present are retained by the curd cubes instead of by the whey.[164]

It has been shown by Brown and Price[82] and by Sanders[171] that the hydrogen-ion concentration is higher in the curd of cheese than in the whey during the period of rapid acid formation, but when the rate of acid development decreases, the pH value of the curd and that of the whey tend to become the same. Sammis et al. found, in working with Cheddar cheese, "that the acidity of whey within curd cubes rises much faster and higher than that of the whey surrounding the cubes," and that "the whey gains most of its acidity from the curd." "Removing part of the whey from the vat soon after cutting does not affect the rate of separation of whey from the curd, but the remaining whey rises more rapidly in acidity as a result of such withdrawal. "Curds made from sweet or from overripe milk retain a larger proportion of moisture than curds from milk of a medium ripeness."

The curd of Cheddar cheese does not show much of a tendency to cohere immediately after it is cut. With an increase of acidity, however, the characteristics of the curd change and, when the whey is removed, the curd particles unite forming a single mass so that the original cubes can not be detected. This fusion of the curd particles is known as "matting" and is an important step in the making of Cheddar cheese. The curd at this stage has changed in a number of physical properties, among them solubility. During the period between cutting and pressing the acidity of the whey normally increases to about 0.7 per cent; the acidity of Swiss cheese whey is usually about 0.10 to 0.12 per cent. In Cheddar cheese, the acidity increases rapidly in the vat and the calcium loss is, therefore, greater than in Swiss cheese. In one day the pH value in Cheddar cheese reaches approximately 4.9 to 5.3[82] and then increases only slightly for one or two months, finally increasing more rapidly to approximately 5.6 to 6.0 at 24 months. Brown and Price[82] found that cheese of inferior quality resulted when the hydrogen-ion concentration was excessive at any given stage in the process.

The acidity changes taking place in the manufacture of Cheddar

cheese are shown in Table LXXXVIII. These figures should be taken only for comparative purposes for reasons already explained.

Table LXXXVIII.—Acidity changes during Cheddar cheese manufacture.

	Acid calculated as lactic	Length of time after cutting
	per cent	hours
Acidity of milk before addition of rennet.........	0.17 -0.20	....
Acidity of whey at time of cutting..............	0.115-0.14	....
Acidity of whey at time of drawing.............	0.13 -0.19	2½
Acidity of whey at time of packing.............	0.24 -0.30	....
Acidity of whey at time of milling..............	0.70 -1.00	4
Acidity of whey at time of salting..............	0.90 -1.10	5½-8

In Swiss cheese the hydrogen-ion concentration increases slightly during the making process; [57] e.g., from pH approximately 6.6 to 6.45, and at least a part of this increase in acidity is due to physico-chemical rather than to bacteriological causes. The acidity increases rapidly while the cheese is cooling and draining on the press, largely due to the activity of organisms of the *Streptococcus thermophilus* type. After several hours the acid increase is carried on by rod-shaped organisms of the *Lactobacillus casei* or *Thermobacterium helveticum* type. The acidity reaches pH approximately 5.0 to 5.3 in one day; during ripening the pH gradually increases, reaching approximately 5.75 in nine months.[95] The rapid acid development of the cheese on the press promotes the drainage of whey from the cheese, and also causes the curd to become relatively firm.[207] At the same time, insoluble calcium becomes more and more soluble.[171] The acidity of the cheese on the press develops more rapidly near the surface than in the inside of the cheese, due to the effect of surface cooling upon the bacterial flora, and excessive acid development at the surface may be detrimental to proper drainage of whey.[88] Klang [88] has recommended keeping the cheese as warm as manufacturing conditions will permit during the first day in order to promote the development of organisms of the *L. casei* type. After about the third week, the acidity decreases more rapidly near the rind than in the center of the cheese.[95]

Orla-Jensen [136, 138] found that increasing the cooking temperature employed in the manufacture of Emmenthal cheese from 48° to 60° resulted in a decrease in the acid in the four hour old cheese in the ratio of 28 to 11. Watson [208] showed that increasing the cooking temperature from 52° to 58° reduced the hydrogen-ion concentration of the cheese on the press very markedly, by inhibiting the growth of lactic acid bacteria.

In Tilsiter cheese the acidity at the rind decreases during ripening until the reaction becomes almost neutral while in the inside of the ripe cheese the pH value is 5.2 to 5.6; when the pH figure in the cheese exceeds 5.7 to 5.82 the flavor is usually defective.[177] Templeton and Sommer [191] found the pH value of good quality process cheese to be between 5.8 and 6.3, and that when the reaction varies outside these limits

the body and the keeping quality are inferior. The gradual decrease in hydrogen-ion concentration in ripening cheese as noted above is due to the destruction of the lactic acid, the formation of weaker acids, including carbonic acid, and the liberation of alkaline products of protein decomposition.

The secondary heating or cooking. The secondary heating is necessary with most of the hard and semi-hard cheeses. In the case of Limburger or of Emmenthal this involves heating to about 55° to 58°. This treatment hastens the expulsion of the whey from the curd, changes its texture, and often alters the bacterial flora. Vas [204, 205] states that the stirring-out process at a high temperature brings about a desired physical condition of the curd which permits the whey to filter off through the cheese particles; the adhesive properties of the granules are so diminished during heating that whey movement between the particles is facilitated when the cheese is on the press. Vas found that the specific gravity of the granules increased from 1.056 to 1.073 during the cooking. Cooking at a high temperature has been found to decrease the moisture content [138, 204] and cause the cheese to ripen slowly.[188] Rennet action is checked, if not wholly inhibited, by the degree of heat usually employed in scalding Swiss cheese curd. Ordinarily the curd is heated slowly at first in order to insure the maximum separation of the whey from the curd, and then more rapidly toward the end. Various physical changes take place in the curd during this period; it becomes firmer, tough, and rubbery, and in the case of Swiss and Parmesan it acquires a certain plasticity.

The maximum temperature of heating varies with the type of cheese being made, the quality of the milk, the quantity of bacterial starter used, and possibly other factors. Orla-Jensen [185] found that a temperature lower than 55° for scalding Emmenthal was not satisfactory.

Homogenized milk in cheese making. Homogenized milk is now frequently used in the manufacture of Neufchatel and Cream cheese, and is said [114] to be beneficial in producing a soft, smooth texture and reducing the fat loss in the whey; in the case of other varieties like Brick, Limburger, and Swiss, this treatment of the milk has given unsatisfactory results. It has been found [24] that the action of rennet is more rapid on homogenized milk than on untreated milk.

Cottage and Cream cheese made from homogenized milk have a finer grain and a closer texture, and consequently a greater percentage of moisture, than cheese made from unhomogenized milk. Sammis [167] found that curds from homogenized milk were fragile and easily broken with curd knives. Limburger and Brick cheese cracked and had a bad flavor when made from homogenized milk. Swiss cheese made from homogenized milk had a bad flavor and was entirely lacking in eyes. It is said that in some cases homogenization seems to produce bitterness and rancidity in Neufchatel and Cream cheese.[116] This effect may be associated with the observation of Dorner and Widmer [52] who found that the process reduces the size of the fat globules and increases their surface

area about ten to thirty times, thus greatly increasing the possibility of fat decomposition due to lipase activity.

Clarified milk in cheese making. Fisk and Price[56] found that there was an improvement in the total score of Cheddar cheese when the milk was clarified. A slight improvement was noted in the body, flavor and texture. They stated that clarification "will sometimes overcome the gas in milk and curd, at other times it will not overcome gas but will change it." Other investigators[42] have found similar improvement, but found the effect of clarification more marked in the case of a good quality milk than in the case of a poor one.

Experiments carried on in the dairy research laboratories of the United States Department of Agriculture in 1921 showed that running milk through a separator before converting it to Swiss cheese resulted in less "oversetting" and in larger and fewer eyes. The body and texture were also improved by this treatment. Twenty-one pairs of cheeses made in a factory, each pair being made from the same batch of milk of which half was centrifuged and half was not, were graded as shown in Table LXXXIX.

Table LXXXIX.—The quality of Swiss cheese as affected by centrifuging the milk used.

	Fancy grade	No. 1 grade	No. 2 grade
	per cent	per cent	per cent
Cheese from centrifuged milk...........	76.2	4.8	19.0
Cheese from uncentrifuged milk	38.1	61.9	None

Table XC shows calculations made from more extensive data on factory cheese not run primarily for experimental purposes.

Table XC.—The quality of Swiss cheese as affected by centrifuging the milk used.

	Number of cheese	Fancy	No. 1	No. 2
		per cent	per cent	per cent
Cheese from centrifuged milk.....	241	77.6	7.1	15.3
Cheese from uncentrifuged milk ..	109	30.3	52.3	17.4

Similar improvement in eye formation by clarification has been noted by other workers. Orla-Jensen[137] found that centrifuging the milk increased the size and decreased the number of eyes, and a similar result was obtained by filtration or by prolonged agitation of the milk. He believed the improvement might be due to the production of a more uniform distribution of gas-forming organisms throughout the milk. Guittonneau[69] and his associates state that clarification may improve eye formation by eliminating some of the centers of bacterial proliferation and breaking up aggregates of microorganisms; they believe that the elimination from the milk of organisms adherent on solid particles, which

are removed by the process, may contribute to the improvement. No conclusive evidence has been offered, however, as to the cause of improvement in eye formation due to clarification. The explanation appears to be the removal of dirt or cellular material. Filtering milk through three layers of absorbent cotton has a pronounced effect on eye formation in the Swiss cheese made from it. Intentional addition of dirt to clarified milk usually causes blow holes to appear in the cheese. Clarification appears to have no effect upon nissler gassy fermentations but seems to have particular value in correcting the defect known as oversetting.

Fat losses in the whey from Swiss cheese are in some cases very slightly increased by centrifuging. Data from 16 pairs of experimental cheese indicate about 2.5 per cent lower yield of green cheese from clarified milk than from milk that was not clarified. The moisture content averaged 0.27 per cent less when clarification was used. In later experiments, data from 36 pairs of cheese show an average reduction of 0.66 per cent in moisture in the green cheese due to clarification. Extended experiments have shown that the decrease in moisture content is not sufficient to account for all of the decrease in yield due to clarification.

Pasteurized milk in cheese making. Pasteurization of milk renders it less readily coagulable than raw milk, so that it was formerly supposed that some special treatment was necessary to correct this condition before using pasteurized milk in cheese making. One procedure recommended was to cool the pasteurized milk to 32° and then to add 2 cc. of 25 per cent $CaCl_2$ solution, 2 to 3 pounds of lactic starter, and 3 ounces of rennet per 1,000 pounds of milk. Sammis and Bruhn[166] recommended the following method: Following pasteurization at 74° the milk is cooled to 24° or lower and sufficient normal HCl solution added to raise the acidity to 0.25 per cent calculated as lactic acid. The milk is then warmed to 30° and 7.5 pounds of lactic starter and 2 ounces of rennet added per 1,000 pounds of milk. By this procedure there is sufficient acid present so that the whey may be drawn immediately. The customary procedure is followed from this point. Cheese made by this process and cured at 16° to 21° for about 100 days gave 4.22 per cent greater yield on the average than raw-milk cheese similarly cured. The average fat loss in the whey was 0.25 per cent for the raw-milk cheese and 0.159 per cent for the pasteurized-milk cheese.

Pasteurization of milk causes a noticeable softening of the curd in milk[155] and in the resulting cheese.[173] The making process is delayed,[87] and the curd is weak and lacks its characteristic solid, granular appearance. The decrease in curd coagulation is said to be accompanied by a decrease in protein in the whey, and one is a linear function of the other;[128, 129] the whey from pasteurized milk cheese filters slowly and leaves a gelatinous precipitate,[128] indicating a change in the physical nature of the milk proteins. The development of hydrogen-ion concentration by starter organisms in the cheese is hastened by pasteurizing the milk;[128, 172, 173] this increased acidity seems to be maintained throughout the ripening period, and, in the case of milk of good quality, a desirable

flavor develops sooner in pasteurized milk Cheddar cheese as compared with cheese from unpasteurized milk.

In spite of the increased development of flavor, Moir [128] found that protein decomposition is relatively slow in pasteurized milk cheese, and that pasteurization may increase the tendency toward bitterness. Pasteurization causes an increase in the yield of Cheddar cheese; the softer curd tends to retain moisture,[78, 87] which, as in the case of Swiss cheese, accounts for at least a part of the increased yield. Certain workers [87] have found that pasteurization of milk increases the fat loss in Cheddar cheese whey, while other workers [78] report no increase in fat loss. Marquardt [114] reported that in making Neufchatel and Cream cheese pasteurization increases the fat loss unless the milk is homogenized. He found that the flavor and keeping quality of the cheese is improved by pasteurization, and recommends a temperature of 180° for 10 minutes for this purpose. In the case of Edam cheese, Van Dam [198] found that the process of ripening and the development of flavor are inhibited as the temperature of pasteurization is increased. It is said [78] that Cheddar cheese made from pasteurized milk is equal in flavor and superior in body and texture to that made from unpasteurized milk.

Pasteurized milk has generally been considered unsatisfactory for the manufacture of Emmenthal or Swiss cheese; [66, 206] Gratz [66] states that the heating causes changes in the casein which adversely affect the formation of eyes, and that there is a tendency for anaerobic organisms to increase and cause defective eye formation and abnormal ripening. By properly modifying the procedure, however, Swiss cheese of good quality has been made from milk pasteurized by the holding process.[59, 217, 220] In the case of milk of poor quality, pasteurization, together with the use of proper starters and a modified making process, is said to produce a decided improvement in the quality of the cheese. These improved methods are largely the result of the work of Frühwald, who has described the procedure as applied to Emmenthal cheese.[61]

Practically all Cream and Neufchatel cheese made on a factory scale and part of the cottage cheese are made from pasteurized milk. As in other varieties, pasteurization improves the yield. It seems probable that pasteurization at high temperatures causes a precipitation of some of the albumin, thus making the product slightly more sticky.

The use of salts and neutralizers in cheese making. Klein and Kirsten [89] added $CaCl_2$ to milk after pasteurizing and were able to obtain fairly good Limburger and other soft cheeses. They used from 100 to 125 cc. of a 4 per cent solution for each 100 kilograms of milk. Sammis and Bruhn [166] obtained favorable results when they used $CaCl_2$ in pasteurized milk for Cheddar cheese. At present the use of salts to correct for changes due to pasteurization is uncommon in this country.

Salts have been added to raw milk used in cheese making, particularly in France. One investigator [195] reported that the use of 1 gram of dry $CaCl_2$ per liter of milk results in more rapid curdling, faster draining, a clearer whey, and a higher yield of cheese. The added calcium evi-

dently appears in the cheese as calcium phosphate. Koestler[92] has recommended the use of small quantities of $CaCl_2$ to cause curdling in certain types of abnormal milk which otherwise fails to curdle.

Price[151] found that the use of $CaCl_2$ in Cheddar cheese increased the yield and caused the retention of milk fat and other solids without producing any decrease in quality. Similar results were obtained by Knaysi and Nelson,[91] who believe that the more complete precipitation of the curd is due to at least two factors: first, the depressing effect of $CaCl_2$ upon the dissociation of calcium caseinate with which it has a common ion; and second, the action of $CaCl_2$ in favoring the formation of insoluble phosphates and citrates of calcium at the expense of phosphates and citrates of sodium and potassium, in which the casein is relatively soluble.

Basic substances have been used to neutralize high acid milk previous to its use in cheese making. Pasteurization usually accompanies this treatment. However, the results appear to be somewhat unsatisfactory and such a procedure can not be recommended.

Loaf cheese. Blending and manufacture of loaf cheese is now carried on extensively with Cheddar and Swiss, and to a less extent with Limburger and Camembert. The process in general[159] consists of grinding the cheese, heating it in steam-jacketed containers to 60° or 70°, and pouring into molds. In the initial stages of heating there is a slight separation of fat, but on the application of more heat the casein becomes plastic and stringy and incloses the other constituents of the cheese, forming a homogeneous mass. Further agitation causes the mass to lose its plasticity and to become of the consistency of heavy cream. It is then ready to be poured into molds. The plasticity of the casein appears to be the most important factor in the process. The method of manufacture, the degree of ripening, the acidity of the cheese, and possibly other factors influence the degree of plasticity attainable in the heated cheese and the length of time that the mass will remain plastic. Once the plastic condition has broken down, it is practically impossible to restore it again. Certain salts, such as those of sodium and ammonium, seem important for the proper emulsification of the product. The quantity of NaCl is an important factor in the destruction of bacteria during the heating, though too much is said to stiffen the product and make it coarse. Robinson believes that in the final stage of the process the fat particles are surrounded by a thin layer of colloidal casein, since it is very difficult to remove the fat from loaf cheese by ordinary methods. The rapid physical changes which the cheese undergoes in this process seem quite in accord with the work of Robertson[158] on the solubility of casein in alkaline solutions.

Cheese ripening. Cheddar cheese passes through a series of physical and chemical changes during ripening which cause the body of the cheese to lose its somewhat tough, elastic, and rubber-like properties and to become soft and mellow. Coincident with this change the insoluble nitrogenous constituents are to some extent changed to a soluble form. Kelly[86] found that the non-protein nitrogen in ripe Cheddar cheese repre-

sented up to 24 per cent of the total nitrogen. A temperature of about 4° is most desirable for curing Cheddar cheese. At this temperature the growth of organisms producing gas and taints in milk is practically suspended and the texture is close and free from holes. At lower temperatures the rate of ripening is considerably decreased. To bring about a more rapid ripening, the cheese is often ripened at 12° to 15°, and then stored at a temperature just above freezing.

The decomposition of protein in cheese ripening is directly related to the numbers of lactic acid bacteria present,[17] and such decomposition is known to occur through the action of enzymes produced by the cells, even after the death of the cells (see p. 325). Bacterial growth and chemical ripening occurs more rapidly in a highly hydrated curd than in a curd containing less water.[168] Of the coagulating ferments, chymosin [197] and pepsin [77] are said to take part in cheese ripening, and of these pepsin is said to be the most important.[223] Barthel and his associates [16] found rennin in numerous varieties of cheese which had been ripening for several months, but none was found in Emmenthal cheese since the high cooking temperature used destroys the rennin enzyme. Certain workers [228] state that the presence of lactic acid stimulates proteolysis; it has been found that neither the rennin enzyme nor commercial pepsin produce proteolysis in the absence of acid, but either is proteolytic when acid is present.[199]

Most kinds of soft and semi-soft cheeses are covered with tin foil, which not only serves to give the cheese an attractive appearance, but also aids in the production of anaerobic conditions, prevents desiccation and the escape of volatile products. Aluminum sheeting has been used to some extent in place of tin foil for wrapping cheeses. Objection has been raised [190] to its use because of the chemical and galvanic action which ensues and often results in the complete disintegration of the wrapper.

Emmenthal cheese becomes softer and more elastic during the early stages of curing. While in the cold room at 13° to 14° the cheese is firm and somewhat brittle. As the cheese takes up salt and is transferred to a room at 22° the curd becomes sufficiently elastic so that a plug of cheese may be wound around the finger, and later in the ripening, the curd tends to become more firm as it loses moisture; [95] during this period eye formation takes place, and when the curd firmness becomes extreme during eye formation, defective checks and cracks and the glaesler defect may form in the curd. Normal eyes are formed as a result of the evolution of gases, principally CO_2, and in abnormal or early eye formation the gas contains relatively large amounts of hydrogen.[84] According to Clark [40] the gas "separates in aggregates whose localities have no necessary relation to the points where the gas is produced," and "a rapid gas formation must tend to the formation of numerous small holes, while slow gas production must admit of the formation of large holes." Following the normal swelling due to eye formation, repeated saltings, and the loss of moisture, the cheese gradually acquires firmness and becomes "set." It is then ready for the final curing at about 13°.

The interior of Swiss cheese is said to be practically anaerobic,[94] and fat decomposition in this type cheese is negligible.[218] There is said to be more proteolysis in the interior of hard cheese than at the rind.[65, 94] The amount of soluble nitrogen in various kinds of hard cheese when ripe is said to be usually at least 30 per cent of the total nitrogen, although it may vary between 16 and 50 per cent.[15]

The rate at which salt diffuses from the surface to the interior of cheese is of considerable importance. The outer portions of Emmenthal cheese contain more salt than the interior, and this is given as the reason[139] why the outer layer contains fewer holes than the central portions. Salt influences the progress of ripening by inhibiting the growth of bacteria[228] and thus decreasing the rate of formation of protein decomposition products;[169, 200] moreover the presence of salt influences the solubility of nitrogenous compounds in the cheese[169, 228] and therefore modifies the texture. Over-salting may produce a hard curd and cause the eyes to form slowly and insufficiently. Orla-Jensen[139] found that 0.5 per cent salt decreased the fermentative action of propionic acid bacteria, which help to produce eyes by fermenting lactates; 2.5 per cent salt practically stopped the fermentation, and 10 per cent completely stopped the growth of the organisms. Salt diffuses into Roquefort cheese very slowly and it is probably several weeks before the salt content of the cheese reaches an equilibrium. Thom[192] states that in mold-ripened cheese "salt restrains the development of *Oidium lactis* in Camembert and shuts it out of Roquefort." Salt "does not prevent the development of molds active in the ripening of Camembert, Roquefort, and in the ripened forms of Neufchatel." Salt inhibits the growth of certain microorganisms more than others; thus the salting of Camembert cheese is said[170] to aid in regulating the proper proportions of the organisms which bring about the ripening. Aside from its preservative action, salt desiccates cheese[161, 200] and gives it an added firmness so that it is not so easily damaged in handling.

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Chapter IX

The Freezing of Milk and Milk Products

General discussion. In studies of the composition-temperature relationships in connection with ice separation in milk products, one may consider milk as a water solution of milk sugar and salts. The suspended proteins and fat modify the relationships through effects upon the rate at which equilibria are established, and affect only to a very slight degree freezing point values.

With lowering of the temperature to the proper degree ice separates from these solutions in the pure state, with the result that there is an increased concentration of the dissolved substances in the unfrozen water. The temperature at which, with cooling, ice can separate from the liquid phase is customarily referred to as the freezing temperature of such solutions. With further removal of heat, after the freezing temperature is reached, the temperature lowers progressively, causing a continued separation of ice and a further increase in the concentration of the unfrozen or liquid phase. This unfrozen phase will of course become saturated with respect to one ingredient after another. At saturation, the separation of these substances may take place, and probably will, at least in part. When the saturation point of the most soluble of the constituents is reached and the others have separated or are separating, theoretically the whole mass can solidify at the existing temperature. This condition probably will seldom be realized in the case of the more concentrated milk products because of the marked tendency of some of the milk constituents to form supersaturated solutions. It must be pointed out, too, that some of the water existing in milk may not play the role of solvent. This is water which may be adsorbed or bound to the protein or the sugar molecules.

Temperature and Effects of Freezing

Milk and cream. Normal milk will, of course, have a freezing temperature lower than that of pure water, due to the effect of the dissolved materials. Winter,[54] in 1896, announced that blood and milk have the same freezing temperature, while Stoecklin[48] pointed out that milk is isotonic with blood and that the freezing point of the two fluids will range from $-0.55°$ C. (31.01° F.) to $-0.560°$ C. (30.99° F.). Mundula,[34] through osmotic pressure measurements, showed that the blood of a woman and her milk were isotonic. Since it has been shown that the milk is isotonic with the blood of the animal from which it is drawn, it follows

that its freezing point is very nearly constant in spite of considerable variation in its composition.

The freezing temperature of normal milk may be taken as $-0.55°$ C. (31.01° F.). This is the value given by Winter[55] for cow's and human milk. He gives the value of $-0.57°$ C. (30.97° F.) for milk of a mare. Since the freezing point of milk is a nearly constant value and since it can be measured with great accuracy, its determination furnishes a method for the detection of water added to milk. Winter's data indicate that for each addition of one per cent by volume of water there is a rise of approximately 0.0055° C. (0.0099° F.) in the freezing point. This is well within the limits of detection since the determination may be accurate to within $\pm$ 0.002° C.

The percentage of water added to a sample may be calculated[1] according to the following formula:

$$W = \frac{100\,(T - T_1)}{T}$$

in which T is the average freezing point of normal milk ($-0.550°$ C.) and T_1 is the observed freezing point of a given sample. A tolerance of 3 per cent is usually allowed in cases of suspected addition of water. The relationships between amounts of added water by volume and the freezing points may also be obtained from a table prepared from the results of Winter.[54] The above formula is not applicable to cream samples.

Hortvet[17] has given a very complete bibliography of the work done upon the freezing temperature of milk, to which the reader is referred.

While the proteins exert some effect on the freezing temperature of milk, this effect is so small as to be negligible for ordinary purposes. Neglecting this effect and employing the molecular weight formula, one may calculate that the milk sugar normally present in milk accounts for a freezing temperature lowering of 0.304° C. (0.549° F.). The difference between this value and 0.550° C. therefore represents the lowering due to the milk salts. This is approximately 0.246° C. (0.443° F.).

Cream will have the same freezing point as normal milk.[9] Until recent years but little seems to have been known concerning the effect of freezing upon milk and cream. This seems surprising when one so frequently encounters speculation as to whether milk and cream are altered harmfully by freezing, or whether or not milk and cream could be preserved by freezing.

Webb and Hall[50] have shown that the effect of freezing upon normal milk is apparently concerned first with the equilibrium of the fat and later with that of the casein. This work has shown that if the freezing time of whole milk is of the order of a few hours, there is an appreciable fat separation when the milk is thawed. In concentrated milks additional percentages of milk solids-not-fat appear to act in a protective capacity with the result that there is progressively less fat separated as the ratio of milk solids-not-fat to fat is increased. Rapid freezing is known to largely overcome fat separation on thawing.

In frozen whole milks the fat separation is followed, upon continued standing for a few weeks, by a separation of the casein. In the first stages of the separation the casein may be resuspended by shaking. In the latter stages the precipitation is irreversible and, in milk of high concentration, gelation results.

If whole milk is condensed to 2½ or 3 times its normal concentration and frozen, it may be held in storage at — 13.3° C. (8° F.) or below and reconstituted at any time within about six weeks to a very satisfactory fluid milk. Higher storage temperatures, increased milk concentration or longer periods of storage tend to produce a gelation of the product, due apparently to changes in hydration of the casein.

Leach and Martin [19] have shown that cream volume is reduced by the partial freezing of milk upon passage over a surface cooler. Webb and Hall [50] found that in creams of about 30 per cent butterfat content that had been homogenized, the mutual adsorption of fat and casein was such that the fat-casein mixture which separated on freezing could be almost quantitatively filtered from the serum during thawing and then resuspended in water. Using this method, with the subsequent removal of the fat from the casein by centrifuging, a water suspension of normal undenatured casein may be prepared. If milk or cream of lower fat content is used the serum separation is not so complete. The precipitation and gelation of casein in frozen skim milk takes place very slowly.

Concerning the storage of frozen cream as a satisfactory method for handling surplus cream, many investigators [37, 39, 40] agree with Mack [29] that the method is useful. The investigators find that the quality depends upon initial quality, the length of time in storage, and the temperature. Holding periods of less than six months and storage temperatures below — 17.7° C. (0° F.) are recommended. The whipping properties of frozen cream are less than normal, but if sugar up to 10 per cent is added to the cream before freezing, ice cream mixes made from this cream whip more easily than those made from normal cream. The addition of gelatin has little or no effect upon the properties of cream that has been frozen. The homogenization of cream before freezing is of no advantage from the ice cream maker's viewpoint.

Work by Baldwin and Combs [2] upon partial and complete freezing of milk and cream has shown that little effect is produced in either product by partial freezing although the complete freezing of cream does alter the emulsion as indicated by the decreased whipping qualities of completely frozen cream and the lowered quality of the butter churned from such cream.

Tracy [45] particularly calls attention to the off flavor produced by freezing cream that has been in contact with copper and iron containers and also calls attention to the off flavors that may be produced by freezing and storing the cream in uncovered containers. These same effects have been observed and studied by Ellenberger.[10]

There seems to be a well-defined prejudice against the use of frozen milk, particularly for baby food. This is probably due to the fact that the

separated fat clumps are not as easily digested as the smaller particles. The fat can, however, be resuspended by homogenization and the product should then be perfectly wholesome. In this connection the work of Munkwitz, Berry and Boyer [35] is of considerable interest. They fed one group of rats upon milk that had been frozen, another group upon milk that had not been frozen. After discussing the changes that occurred in milk upon freezing they conclude: "The results with rats as shown by growth rates, calcification of femur bones, and physical condition did not indicate that freezing impaired the nutritive value of milk."

Evaporated milk. The minimum standard for evaporated milk calls for a concentration of 25.5 per cent total solids, 7.8 per cent fat and 74.5 per cent water, or a milk solids-not-fat concentration of 17.7 per cent. Evaporated milk will then have, on a water basis, a concentration of solids about 2.32 times as great as that of normal milk, and its freezing point will be correspondingly lowered. The calculated freezing temperature is — 1.34° C. (29.6° F.). Evaporated milk of this legal minimum composition will be slightly supersaturated to lactose at a temperature of 0° C. (32° F.).

If evaporated milk is held in a frozen state for a short period in storage, a slight amount of fat separates and there is an indication of the presence of a small amount of denatured casein. In other respects the milk is normal in its properties. As in the case of milk and cream the extent of fat liberation and the denaturation of casein is dependent upon the rate of freezing and the length of time it remains in a frozen condition (see pages 252 and 253).

Sweetened condensed milk. The legal minimum concentration of a sweetened condensed milk is 8.0 per cent fat and 20.0 per cent milk solids-not-fat, and it is customary to use at least 42.0 per cent cane sugar. This leaves a water concentration of 30 per cent. Such a milk would be supersaturated to lactose at ordinary temperatures and supersaturated to both sucrose and lactose at its theoretical freezing temperature of — 14.96° C. (5.07° F.) which is calculated on the basis that neither sugar has begun to crystallize. If both sugars should crystallize it would be possible for the milk to be frozen solid at approximately this same temperature. It is quite apparent that at low temperatures, excepting those attained in the severest of winter months, sweetened condensed milk will not become frozen.

Butter. There is no available information concerning the temperature of ice separation in butter. It is evident that the freezing temperature of the liquid phase of unsalted butter will not be lower than that of milk, but the presence of the normal amount of salt will lower the actual freezing temperature quite markedly.

Freezing apparently does not harm butter in any way, it being quite the usual procedure to hold it at 0° F. or lower in storage. The United States Bureau of Dairy Industry has actually exposed butter samples to freezing by means of solid carbon dioxide for several days without producing any noticeable effect upon the quality of the butter.

Cheese. There is available very little information regarding the freezing points of various cheeses. The freezing points of a number of miscellaneous cheeses, as determined in the laboratories of the Bureau of Dairy Industry,[40] are given in Table XCI.[15] The processed cheeses were about six months old. The other cheeses were about a year old.

Table XCI.—The freezing points of the common varieties of cheese.

Variety of cheese	Freezing point	
	° C.	° F.
Cottage	−1.2	29.8
American (processed)	−6.9	19.6
Limburger	−7.4	18.7
Picnic Swiss (processed)	−8.1	17.4
Brick	−8.7	16.3
Swiss (imported)	−9.6	14.7
Swiss (domestic)	−10.0	12.0
Cheddar	−12.9	8.8
Roquefort	−16.3	2.7

Sommer[41] has found the freezing points of fifteen different Cheddar cheeses to range from − 4.3° C. (24.3° F.) to − 14.3° C. (6.3° F.).

When a cheese is first dipped from the kettle it consists of particles of curd (fat and protein) wet with whey. Theoretically the freezing temperature at this time would be the freezing point of the whey (− 0.55° C.). The moisture content of a cheese does not vary markedly with the ripening. Since the freezing point decreases with curing it seems evident that water must be removed from its role as solvent, probably by combination with the proteins of the cheese. This is a point which has not yet been investigated, but one which should be of considerable interest.

Van Slyke and Publow[48] state that cheese placed in a room at a temperature of − 15° C. (5° F.) was rapidly frozen hard. After a time the ends and sides appeared to be lumpy, due to the expansion of the frozen water in the cheese. After remaining in the frozen condition for six months the cheese was slowly thawed and examined. When freshly cut the appearance was normal, but the surface dried out more rapidly than did the surface of normal Cheddar cheese. The body was as crumbly as that of a cheese deficient in water. Little or no ripening had taken place and the flavor did not resemble that of normal cheese. The frozen cheese also showed a mottled appearance. Sommer,[41] however, in experiments upon Cheddar cheese, found that freezing caused no injury to the flavor although it caused the texture of the cheese to become crumbly. On storage at favorable temperatures, after freezing, the cheese texture recovered, in some cases apparently completely.

Powdered milk. Dried or powdered milks have so low a moisture content that for practical purposes they can not be considered to have a freezing temperature. The moisture present is largely in a bound form and hence in a state not readily frozen, even at low temperatures.

Ice cream mixes. The freezing temperatures of ice cream mixes are

dependent upon the same factors that control the freezing temperatures of milk, namely the soluble constituents, and will vary with variations in the composition of different mixes. Of the constituents—namely, fat, milk solids-not-fat, cane sugar and gelatin—the sugar exerts the greatest effect in lowering the freezing point.

Work upon the freezing equilibrium of ice cream mixes has been done by Cole [4] who succeeded in separating the liquid phase from frozen ice cream by filtration under pressure. Leighton [21] has shown that it is possible to calculate with considerable accuracy the freezing point of ice cream mixes upon the assumption that the milk salts exert their normal molecular lowering and that the combined sugars (lactose and sucrose and possibly dextrose) exert the same abnormal lowering characteristic of cane sugar. If dextrose is used in the mix it may be considered that one part dextrose is equivalent to 1.73 parts cane sugar in its effect upon the freezing point.

It is interesting to observe that if, as is usually considered, the abnormal lowering of the freezing temperatures of cane sugar-water mixtures is due to hydration of the sugar molecules whereby water is removed from its role as solvent, the milk salts should give this same abnormal lowering in cane sugar solutions since less water would be available to dissolve them. Experiments seem to show that this is not the case. The method of calculation must be considered as empirical. The method assumes that the mix is made up from normal milk products and that none of the lactose or milk salts has been removed; in other words, that the ratio of salts to lactose is that of normal milk. No consideration is given to the effect of flavoring materials.

It has been shown that the apparent molecular weight of the salts of milk is 78.6.[21] With this as a basis, and neglecting the effect of fat and gelatin, the relationship may be expressed as follows:

$$\frac{\text{M. S. N. F.} \times 2.37}{\text{Pts. } H_2O} = (A) = \text{Salt lowering in degrees C.}$$

$$\frac{(\text{M. S. N. F.} \times 0.545 + \text{pts. sucrose})\ 100}{\text{Pts. } H_2O} = \frac{\text{Parts total sugar to}}{100 \text{ pts. } H_2O}$$

If the total sugar concentration in a mix is calculated by means of the equation given above the freezing point depression (B) in degrees Centigrade due to the sugar may be obtained from Pickering's table,[36] which follows, and the magnitude of the freezing point depression determined by adding A and B.

The total solids content of ice cream will usually be found within the limits of from 30 to 40 per cent. The sugar and gelatin content of ice cream is fairly constant. Greater variations may occur in the proportions of fat and of milk solids-not-fat. As a rule the fat content of the ice cream to be manufactured is of a chosen value and the proportion of milk

Table XCII.—Freezing point lowering of cane sugar solutions. Pickering's Data.

Parts cane sugar to 100 parts water	Per cent cane sugar	Lowering ° C.	Lowering due to 1 part cane sugar
3.59	3.47	0.21	0.05
6.85	6.41	0.40	0.05
10.84	9.78	0.65	0.06
15.83	13.67	0.95	0.06
19.80	16.53	1.23	0.06
22.58	18.42	1.37	0.06
25.64	20.41	1.58	0.06
28.51	22.19	1.77	0.06
32.22	24.37	1.99	0.06
35.14	26.00	2.15	0.06
37.86	27.46	2.33	0.06
43.72	30.42	2.71	0.06
45.62	31.33	2.82	0.07
50.02	33.35	3.13	0.07
54.74	35.37	3.47	0.07
59.46	37.29	3.81	0.07
64.55	39.23	4.22	0.07
69.74	41.09	4.60	0.07
75.91	43.15	5.07	0.07
82.35	45.16	5.65	0.07
88.67	47.00	6.11	0.07
95.94	48.97	6.76	0.07
102.70	50.65	7.38	0.07
111.30	52.67	8.06	0.07
121.00	54.75	9.02	0.07
131.60	56.82	9.93	0.07
143.10	58.86	10.90	0.07
153.80	60.60	11.69	0.08
165.60	62.35	12.72	0.08
181.70	64.49	13.80	0.08

solids-not-fat is adjusted accordingly. The calculated freezing temperatures of a number of mixes are given in Table XCIII.

Table XCIII.—Calculated freezing points of representative ice cream mixes.

Fat	Milk solids-not-fat	Sugar	Gelatin	Water	Freezing temperature	
per cent	per cent	per cent	per cent	per cent	° C.	° F.
8.00	11.50	13.00	0.50	67.00	—2.20	28.0
8.00	12.50	13.00	0.50	66.00	—2.29	27.9
8.50	12.00	13.00	0.50	66.00	—2.26	28.0
9.00	11.50	13.00	0.50	66.00	—2.29	27.9
10.00	10.50	14.00	0.50	65.00	—2.20	28.0
12.00	8.50	14.00	0.50	65.00	—2.09	28.2
12.00	9.50	14.00	0.50	64.00	—2.21	28.0
16.00	7.50	14.00	0.50	62.00	—2.08	28.2
18.00	7.50	14.00	0.50	60.00	—2.15	28.1
18.00	5.50	14.00	0.50	62.00	—1.91	30.3

It is apparent that in spite of a rather wide variation in the relative fat and milk solids-not-fat content there is no great variation in the freezing temperature. This is due to the fact that the percentage of sugar, which

exerts the greatest effect, is not varied greatly and that the change in the water percentage of a mix as the fat content is increased is not marked.

A normal mix containing 12.00 per cent milk fat, 14 per cent sugar, 10 per cent milk solids-not-fat, 0.3 per cent gelatin and 63.7 per cent water will have a freezing temperature of $-2.26°$ C. (28.0° F.). It contains lactose in the concentration of 8.55 parts per 100 parts of water, and will therefore not become saturated to milk sugar until a temperature slightly below that of the freezing point is reached. If the lactose has not crystallized, 50 per cent of the water will be frozen to ice at a temperature of $-4.67°$ C. (23.6° F.) and 75 per cent will be frozen at $-10.52°$ C. (13.06° F.). At a temperature of approximately $-13.9°$ C. (7.0° F.) the cane sugar can crystallize and may do so to a certain extent.

Little or nothing is known of the solubility of the milk salts at these temperatures. They probably separate gradually as the mix becomes more and more concentrated.

A problem of considerable interest and importance is that of the rate of crystallization of the various soluble components from their saturated solutions. In the presence of protective colloids the rate may be retarded considerably and under certain conditions prevented entirely. The relation of various factors and conditions to this phenomenon needs further study.

Ice Cream

The effect of freezing on milk, cream, cheese and butter is usually considered in connection with the properties of the thawed product as compared with the properties of the unfrozen product. In the case of ice cream, however, it is the properties of the frozen product that are of primary importance. Since the effects of freezing of ice cream are dependent upon the properties of the mix, as well as upon the actual freezing processes in themselves, to understand the effects of freezing, it becomes appropriate to consider the physical properties of ice cream and methods of measuring them. Following this, ingredients and the steps of ice cream manufacture will be discussed in relation to the physical properties of the mix and of the frozen product.

The physical characteristics by which different ice creams are usually distinguished are flavor, texture and body. These characteristics are usually determined by taste alone and for that reason are difficult of precise or accurate description. Flavor is dependent upon the ingredients used and their quality and is, of course, incapable of evaluation except by personal judgment. Texture has to do with the size of the individual particles and air cells that make up ice cream and is usually expressed in terms of smoothness, coarseness, graininess, etc. This property is, in all probability, dependent also to some extent upon body, which has to do with the mass properties of ice cream usually expressed in terms of weakness or heaviness, and must be closely related to the rheological properties of the frozen mass.

For ice cream to be considered of good quality it must have a smooth

texture, which usually is the result of fineness of grain and fineness of air cell size. Ice cream can be of such fine grain however that it cloys. This means that between the ice cream of coarse texture and that of exceedingly fine texture there is a considerable range of texture variation which would be generally acceptable. The same is true of body.

The texture of ice cream has been studied microscopically by Dahlberg [6] and Cole.[5] Both found a correlation between crystal size and texture, but failed to find any simple relationship between texture and size of air cells.

It seems evident that the body and texture of ice cream must be related in some way to measurable physical properties of the mass. Realizing this and also the need for methods of evaluating these physical characteristics quantitatively, Leighton and Leviton [25] have used the sagging beam method of Trouton [46] to determine the plastic properties of ice cream.

Working at a temperature of − 8° C. (17.6° F.) they found ice cream to have such a low yield value that for all practical purposes it could be considered a viscous, rather than a plastic material.

Their work indicates that the absolute viscosity of an ice cream can not be considered as a direct measure of quality. They find, however, that when any factor such as gelatin content and fat content is varied in a series of ice creams, the viscosity-concentration curve for such a series does follow the changes in the physical properties of the ice creams. In such a series the ice cream of the most desirable quality is invariably one whose viscosity is just above the minimum for the series; or, when no minimum exists, of such concentration of material that the viscosity tends to show a sharp increment in value with further concentration increases.

This may be illustrated by their results dealing with milk fat variation as shown in Figure 33.

As indicated, an increase in the fat content first increases the viscosity of the ice cream, then decreases it markedly to a minimum between the ratios of 12, 15 and 18 parts fat and then increases the viscosity at 21 parts. Of these ice creams that containing 18 parts fat had the best body and texture as determined by taste. It is evident that with increasing fat content the viscosity of ice cream is first increased through a binding or mass effect of the milk fat. Then with increasing quantity of fat the protective effect on the ice crystals or a lubricating action results in lower viscosity simultaneously with better texture. Finally the mass effect of the large amount of fat becomes evident and the viscosity increases. The results indicate that three different ice creams of the same absolute viscosity but of widely varying quality are possible. The conclusion is, therefore, that absolute viscosity is not a direct measure of quality, although viscosity variations do follow changes of quality in a logical manner.

It seems logical to consider next the ingredients which are used in ice cream and then to follow through the various steps of manufacture, showing their action upon the materials used in ice cream and their effect upon the physical properties of the mix and of the finished product.

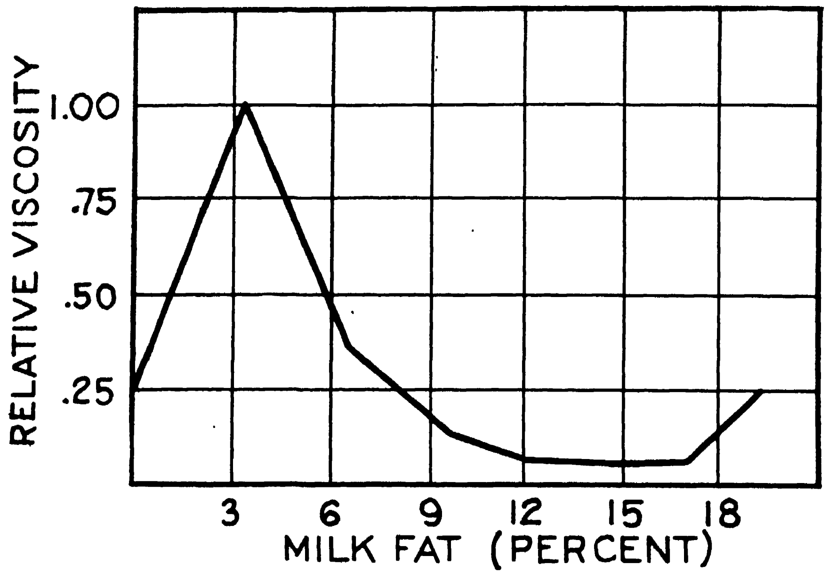

FIG. 33.—Effect of milk fat content upon the relative viscosity of ice cream.

Ingredients. The materials used in the manufacture of ice cream are milk fat, milk solids-not-fat, sugar, flavoring material and possibly a stabilizer or improver. Milk fat imparts a richness of flavor to ice cream as well as improving texture and contributing to the attainment of desirable body. The milk solids-not-fat portion of ice creams is necessary for whipping and contributes to body and to a lesser extent than milk fat to flavor. The freezing point of the mix is lowered by the presence of the milk sugar and salts present in this form of solids. Sugar is used primarily as a sweetener but lowers the freezing point of the mix markedly, thus preventing ice cream from being frozen solid, even at very low temperatures. Were it not for the lactose and sucrose in an ice cream the mass would freeze solid at a comparatively high temperature and the product would be entirely lacking in those desirable qualities associated with ice cream. The flavoring material of ice cream usually does not affect the physical properties of the ice cream markedly unless it adds sugar, as in the case of sweetened-fruits, whereby the normal freezing temperature of the mix is altered. Nut meats or other finely divided solids may also act as nuclei for the crystallization of lactose, giving rise to sandy ice cream. The improvers or stabilizers are employed either to improve body and texture or to stabilize the finished ice cream by making it more resistant to melting.

Mixing the ingredients. In the preparation of the ice cream mix it is customary to weigh out the ingredients in such a way that the final mix shall have a predetermined composition. The order of mixing the ingredients is usually the procedure that permits of easy solution of the

sugar, gelatin, milk powder, flavoring, etc. As far as is known, the order of mixing has no bearing upon the physical properties of the mix nor upon the properties of the finished ice cream, provided that all the ingredients are present during the pasteurization process which follows. Some experimental work has been done to show the effect of the addition of gelatin and sugar before and after pasteurization and homogenization, but the effects are not marked enough to warrant any departure from the usual procedure.

Pasteurization. The solution of the ingredients is facilitated by raising the temperature of the mass until pasteurization temperature 63° C. (145° F.) is reached, where it is the custom to hold the mix for one-half hour. Higher temperatures and shorter periods of holding may be used. While the purpose of the pasteurization is to rid the mix of pathogenic organisms, the process does have some effect upon the physical properties of the mix.

The work of Dahle and his associates [7, 81] upon pasteurization, in agreement with experiments by others, has shown that higher temperatures reduce the degree of fat clumping with a resulting decrease in viscosity, hasten freezing and increase overrun. They report little effect upon the quality of the finished ice cream except for a slight advantage in smoothness in favor of the lower pasteurization temperatures. Erb,[11] however, has reported that low pasteurization temperatures, particularly in conjunction with high homogenization pressures, make for graininess in the melted ice cream.

There is also evidence that some of the calcium and phosphorus of the ice cream mix is rendered insoluble by pasteurization, resulting in a raising of the freezing point by from 0.001 to 0.002° C.[80]

Homogenization. The word "processing" is defined as "an attempt to improve the ice cream mix by mechanical means," and refers to emulsification, viscolization and homogenization. The main effect sought is that of reducing the fat to a fine degree of division and high degree of dispersion, thus preventing its churning tendency during freezing and also imparting smoothness to the frozen product.

The viscosity lost by pasteurization is usually more than regained by homogenization. The viscosity increase in ice cream mixes due to homogenization has been shown by Mortensen [82] and later by Reid and Moseley [38] to be in part at least the result of the aggregation of the disperse fat globules to form clumps. The extent to which this phenomenon occurs depends upon the proportion of ingredients used in the mix and the pressure of homogenization. The action is favored by increased fat content and seems to occur most markedly, not when the greatest pressures are used, but at certain intermediate values. The reasons for the variations in clumping at different pressures of homogenization are not well understood. A second or third homogenization reduces the viscosity of the mix by breaking up the clumps.

The increase in viscosity due to the formation of clumps may be attributed to the entrapping of a portion of the continuous medium in the

interstices of the clumps. This enclosed portion then functions as if it were fat in its effect upon viscosity. The results of Troy and Sharp [47] on the rate of rise of fat clusters in milk indicate that these clusters behave as spherical aggregates.

Treating the combination of ingredients, other than fat, in cream and ice cream mixes, as the continuous medium, and considering the viscosity formula, derived by Taylor [44] on the basis of theoretical hydronamics, for dilute suspensions, as valid at low fat concentrations, Leviton [27] has derived an empirical formula expressing the relationships between the viscosity of a mix free from fat clusters, and that of its continuous medium. The Taylor equation, and the empirical formula based on the equation are, respectively

$$\eta = \eta_0 \left(1 + 2.5 \frac{\eta' + \frac{2}{5}\eta_0}{\eta' + \eta_0} v\right)$$

$$\ln \frac{\eta}{\eta_0} = 2.5 \frac{\eta' + \frac{2}{5}\eta_0}{\eta' + \eta_0} \left(v + v^{5/3} + v^{11/3}\right)$$

in which

η = viscosity of the emulsion
η_0 = viscosity of the continuous medium
η' = viscosity of the dispersed phase
v = ratio of the volume of the dispersed phase to total volume.

If the apparent volume of the fat phase, per unit volume of mix, in a mix containing clusters, is calculated by means of equation,[2] from a knowledge of the viscosity of the mix and that of the continuous medium, then the difference between the apparent volume and the measured fat volume is equal to the volume of the continuous medium associated with the fat clusters per unit volume of mix, and the ratio between this volume and the measured volume of fat may be designated as a clumping index.

A clumping index, so calculated, undoubtedly expresses with greater accuracy the degree of clumping within a cream or ice cream mix than any microscopical count or particle size measurement. However, these equations cannot be construed to apply, without further experimentation, to such mixes as may exhibit marked plastic or elastic properties, as for example, a mix containing gelatin in which a gel exists at the time of measurement.

With the aid of this method Leviton [26] has investigated the relationship between clumping and the increase in the viscosity of cream and of ice cream mixes, save those containing gelatin and has shown that within the limits of experimental error the viscosity increase due to homogenization may be attributed solely to clumping.

Hening [14] found that in general there is no great difference in the effect of gelatin added after homogenization as compared with that of gelatin added before processing the mix. However, the presence of sugar during the homogenization process did markedly increase the clumping of the fat particles, increasing the viscosity of the mix and interfering

with the attainment of overrun. The quality of the ice cream was not markedly different in either case, although the ice cream made from mixes homogenized without sugar was perhaps smoother.

Aging. After homogenization it is the custom to cool the ice cream mix to a temperature of approximately 4.4° C. (40.0° F.) and to hold the mix at this temperature for a period of time extending from a few hours to a day. The object of aging is to improve the whipping capacity of the mix and the body and texture of the finished ice cream.

Dahle, Keith and McCullough [8] show that unaged mixes are likely to fail in attaining the desired overrun during the freezing process, and that aging decreases the time required to obtain a desired overrun and increases the amount of overrun that would otherwise be obtainable. They also found that the most pronounced improvement was to be obtained during the first four to twelve hours of aging. This latter observation has been corroborated by Hening.[15]

While, as has already been stated, it is usually the custom to cool the homogenized mix immediately to a low temperature, Wright [56] and Mueller and Frandsen [38] have shown that certain desirable results can be obtained if the mix is held at 20° C. (68° F.) for about four hours. The efficiency of the gelatin was increased whether this was followed by aging at a lower temperature or not. Such aging lowered the viscosity of an ice cream mix slightly, but there was a great increase in viscosity if aging was continued at 3.3° C. (38° F.). Unless lower quantities than normal of gelatin were used under such conditions, the ice cream melted slowly and had a curdled appearance. Aging at the higher temperature had no effect unless gelatin was present.

The most noticeable effect of aging upon the ice cream mix is to increase viscosity. The structure of the ice cream mix so obtained can, however, be beaten down, and Leighton and Williams [22] have shown that the normal beating in the freezer will reduce the structural viscosity to a constant value. This viscosity value they call "basic viscosity" and point out that this must be the viscosity value that influences the process of freezing if there is to be any correlation found between viscosity and the other phenomena incident to the freezing process. They also show that there is a logarithmic relationship between the viscosity and the total solids concentration of a mix, which follows a modified form of the empirical equation given by Arrhenius, namely—that $\log \eta = \Theta C + k$ where η is the viscosity value in centipoises, Θ and k are constants and C the concentration in parts total solids to 100 parts of water. Other data [23] show that within the temperature ranges usually encountered in ice cream work this basic viscosity is inversely proportional to the temperature. It has been shown, therefore, that ice cream mixes exhibit both a structural and a basic viscosity. The basic viscosity is a true viscosity—the structural viscosity is a plastic property.

From the foregoing it will be seen that aging is intimately connected with the gelation of the mix. It is a period of time in which gelation may take place and during which fat particles may solidify and also during

which any adsorption equilibrium, that may exist between fat, gelatin and protein, is reached.

Freezing and whipping. The action of the ice cream freezer is two-fold. The ice cream mix is partially frozen and at the same time air is whipped into the mass. Since in the freezing process several factors are changing at the same time, it is difficult to picture the exact relationships between variations in ingredients and methods of processing in their effect upon the freezing process. And it is true that these relationships have

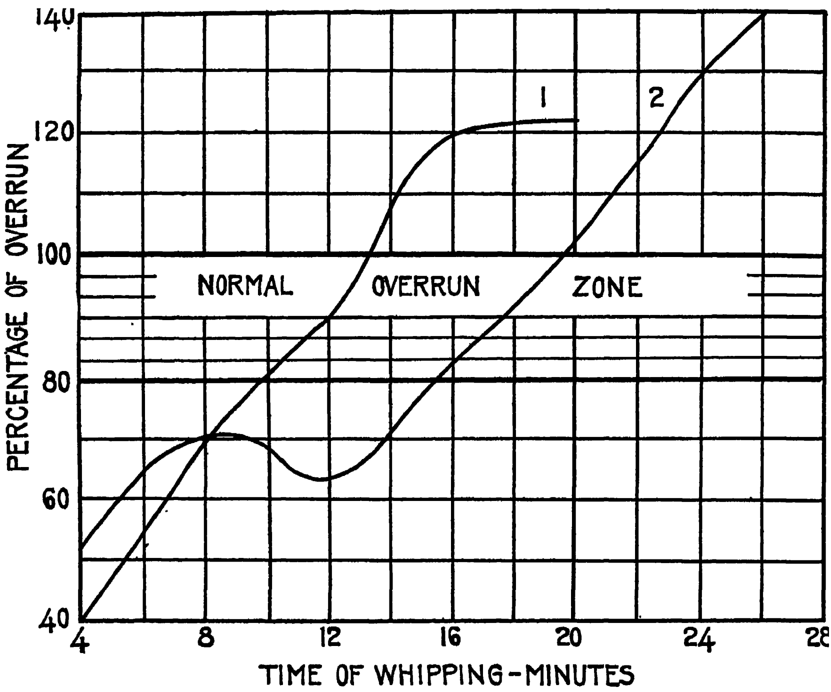

FIG. 34.—Typical overrun curves for ice cream. Mix 1 has 16 per cent fat, 6 per cent milk solids-not-fat, 16 per cent sugar, 0.25 per cent gelatin. Mix 2 has 12 per cent fat, 10 per cent milk solids-not-fat, 16 per cent sugar, 0.25 per cent gelatin.

not been satisfactorily worked out. During the freezing process, not only is the temperature lowered, but with the separation of ice the concentration of the unfrozen portion of the mix increases and there are changes in the physical properties of this unfrozen portion due both to temperature and concentration changes which proceed simultaneously.

The expansion, or increase of volume, of ice cream in the freezer, due to the incorporation of air, is technically referred to as "swell" or "overrun." The result is a fine grained palatable product which would otherwise be soggy and uninviting. The general nature of the volume change is indicated in Figure 34.

The ice cream maker usually desires a mix which may be whipped as

rapidly as possible to the desired overrun. In considering the whipping capacity of the mix, therefore, this property is commonly considered as being made up of two factors, (1) the rate at which the volume increases under given freezing conditions and (2) the amount of air that can be whipped into the frozen mass. The size of the air cells is also of importance since it is usually considered that the smaller the cells the better the texture of the ice cream.

Sommer [42b] has pointed out that the milk solids-not-fat and the gelatin are the ingredients of ice cream which permit of the whipping in of air. Sugar and water do not whip, water and sugar and gelatin do, and so do milk solids-not-fat and sugar and water. A small amount of fat, as minute a quantity as 0.25 per cent, will materially reduce the overrun obtainable in water-gelatin or water-milk solids-not-fat mixtures.

The effect of fat as a foam depressant has been considered by a number of investigators and a number of explanations have been advanced to account for the phenomenon. Leviton [27] investigated experimentally the accuracy of these explanations and came to the conclusion that none were entirely satisfactory. A more satisfactory explanation suggested by him would attribute the destruction of the foam by fat to the spreading tendency of the fat whereby, in very thin lamellae, a portion of the lamellae are wholly displaced by a fat film. Since the fat film is comparatively unstable, the lamellae would be weakened by the presence in them of these areas of fat.

If too little air is incorporated in ice cream the mass is soggy, if too much air is incorporated the ice cream becames too fluffy and lacks in body. The latter condition may be referred to as "dead whip" and may occur as a result of prolonged whipping at too high a temperature.

The amount of overrun which occurs in ice cream manufacture ranges ordinarily from 40 to 100 per cent. It may, however, reach and even exceed 150 per cent though such instances are not of frequent occurrence.

As indicated in Figure 34, mixes of different composition may behave differently in the freezer. In the case of Mix No. 1 a high overrun is attained rapidly and in order to reduce this overrun the brine flow would have to be maintained until the mix became cool enough so that the high overrun would be destroyed. In the case of Mix No. 2 the initial overrun of but 70 per cent is attained when in all probability the mixture became so cold that overrun decreased. In such a case the brine must be turned off and whipping continued until the desired overrun is reached.

From the theoretical point of view a high viscosity and a low surface tension should favor the attainment of overrun. A high viscosity should favor the retention of air and a low surface tension should facilitate its incorporation. As a matter of fact, while these properties do, in general, have the expected effect, no set of data gives results completely in accord with theory.

A few unpublished results by Leighton and Williams upon the basic viscosity of ice cream mixes, in relation to whipping capacity, seem to indicate that in certain cases high viscosity favors, and in other cases

hinders overrun. A high viscosity usually increases the time required to attain a certain volume increase.

In an attempt to get a better insight into the physical factors which would influence foaming or whipping Leviton and Leighton [26] have made an extended investigation of the surface properties of milk serum solutions and of such solutions containing minute amounts of fat. They have found no relationship between foaming capacity and the following surface properties: static and dynamic surface tension, surface viscosity and the degree of protein adsorption at the air-serum interface.

In addition to composition and viscosity considerations, the rate and length of time of freezing and the rate of whipping will affect the degree of overrun. Composition is of primary importance and since commercial mixes used in the industry vary greatly in this respect the potential whipping values as well as those actually obtained vary widely.

Sugar may be classed as one of the major factors affecting overrun in spite of the fact that sugar solutions as such do not whip. This is brought about by the marked effect sugar has upon the freezing point relationships of ice cream mixes and by the effect of sugar upon the viscosity of such mixes. To increase the concentration of this constituent is a principal means of retarding abnormal swell.

While gelatin makes possible the whipping of sugar solutions that would otherwise not whip, when it is used in ice cream in the presence of milk solids-not-fat and fat, it is usually considered as a deterrent of overrun through its effect in markedly increasing the viscosity of the mix. It is seldom used in greater quantities than one-half of one per cent but even this small amount seems to prolong the time of whipping that may be necessary to obtain a desired overrun as well as to decrease the maximum overrun that could be obtained. The results of Isenberg and Baer [18] indicate that usually improvers do not materially affect overrun.

While the amount of albumin in ice cream is very small, there is sufficient evidence [53] to show that this substance may be an important factor in increasing overrun. For instance, the overrun curves obtained in a comparison of two mixes, one containing a moderate quantity of egg albumin and the other made up without additional albumin, showed a maximum difference of 13 per cent in favor of the former. The development of small amounts of lactic acid in ice cream mixes may have a pronounced effect in increasing the overrun.

As to the actual freezing process, ice of course forms upon the surfaces of the freezer and is scraped off by the scrapers. Rapid freezing gives fine crystals and it is also essential that the scraping blades be sharp, otherwise the crystals may be coarse. The effect of fluctuating the temperature during freezing has not been studied thoroughly, but data by Leviton and Leighton [26] seem to show that such fluctuations may cause coarsening of the ice crystals in much the same way as it does in the hardening room or in the cabinets from which the ice cream is dispensed.

Hardening. In the hardening room, to which the ice cream is taken with not more than half the water frozen to ice,[21] the freezing proceeds

slowly. The temperature at the walls of the can drops rapidly, but it may be at least 24 hours before the center of the can has cooled to the temperature of the hardening room and from five to eight hours before it even approximates the temperature of the surrounding air. From this it would be expected that the ice cream from the center of the can might be markedly different in quality from that near the outside of the can. Actually this does not appear to be so. For large ice crystals to form in the outer layers, unfrozen water would have to diffuse out from the center. Due to the high viscosity of the ice cream, such diffusion apparently does not take place. When the ice cream enters the hardening room, it is full of a tremendous number of ice crystals which act as centers of crystallization for the ice that is yet to form. It can be shown that if all the ice that forms in the hardening room is deposited uniformly upon crystals already present their diameter cannot be increased by more than 1.6 times. This is, of course, calculated on the basis of spherical particles. It is evident, then, that if the freezing operation has been conducted in such a way that small crystals have been formed, their increase in size in the hardening room will not greatly affect the texture of the ice cream. However, if temperature fluctuations are allowed to occur, either in the hardening room or in dealers' cabinets, upon warming the smaller ice crystals will melt, upon refreezing this water will be deposited as ice on the larger crystals and soon a coarse grain will develop through the presence of large individual crystals or through their agglomeration to form flakes.

Sandiness. The gritty condition of ice cream caused by the crystallization of the milk sugar is known as "sandiness." Zoller and Williams [57] were the first to isolate pure lactose crystals from ice cream and thus proved that this was the cause of the defect. The factors favorable to the separation of lactose from solution are discussed elsewhere in this book. It will suffice to say that in an ice cream mix of which the milk solids-not-fat content is less than from 10 to 11 per cent, "sand" will seldom be encountered. The condition which seems to be very favorable for its occurrence is brought about by the melting and refreezing of the ice cream that frequently happens in dealers' cabinets. All ice cream in a frozen state is supersaturated to lactose, and probably the only reason why sand is not of more common occurrence is that at low temperatures lactose crystallization is very slow. The factors influencing the crystallization of lactose have been studied by Leighton and Peter [20] and, more recently, by Whittaker [52] and Herrington.[16]

In an effort to permit of the utilization of a greater amount of milk solids-not-fat in ice cream, Leighton and Leviton [24] have developed a method for the removal of a considerable proportion of the milk sugar from concentrated skim milk. They have shown that the viscosity of a highly concentrated skim milk solution can be lowered by the diluting effect of cane sugar that may be present, without markedly altering the lactose solubility unless it be to decrease it. The viscosity of such a milk is low enough to permit lactose to crystallize and to permit of its separa-

tion from the fluid. Applying the data in their paper, Webb and Williams [51] have shown that it is commercially feasible to separate as much as 70 per cent of the lactose from condensed skim milk. Filtration of the sugar from the liquid phase is difficult but can be accomplished with suitable equipment. Such a milk will permit of the utilization of from 2 to 3 per cent more milk solids-not-fat in ice cream without danger of sandiness. If higher concentrations are used a salty taste is noticeable, although greater amounts might be used in the more highly flavored ice creams.

Similar results may be obtained by the use of sodium caseinate as a source of milk solids-not-fat in ice cream. This casein is, however, denatured.

Gray [18] has shown that milk powder, which normally contains the milk sugar in an impalpable form, may be added to ice cream during the freezing process and that under such conditions the lactose does not go into solution and therefore does not cause sand. In this way the milk solids-not-fat content of ice cream can be safely raised above normal.

Improvers. Improvers are those substances added to ice cream to improve the body and texture of the finished product. Gelatin is to be classed as an improver but is unique in that all the other substances of this class may also be classed as gelatin substitutes. Aging is necessary if gelatin is used in the ice cream mix. When other improvers are used the ice cream maker usually seeks to obtain the same effects produced by gelatin without the delay incident to the aging process.

Frandsen [12] lists the different materials that have been used as improvers and stabilizers, stating that the most common are starch, egg-albumin, milk solids, gum, gelatin and powders prepared from a mixture of two or more of these. Isenberg and Baer [18] point out that improvers retard crystallization to a noticeable extent in mixes containing 34 per cent total solids or less and that they are unnecessary and undesirable in the manufacture of high-grade ice cream. They, of course, simply considered those substances to be classed as gelatin substitutes as did Lucas and Gould [28] who investigated gum tragacanth, gum arabic, agar agar, Col-Ace, Kelco Gel, Krabyn, Lakoe A, Creamthick, pectin, soluble casein and sodium caseinate. In some ice creams there was a tendency to whey off when a number of these substances were used. None affected the surface tension of the mix, one or two increased the viscosity of the mix slightly, and in one or two instances whipping was more rapid. While Lucas and Gould draw no conclusions, a survey of their data would lead one to think that they probably agree with Isenberg and Baer [18] in their statements listed above. Caulfield and Martin [3] experimented with Krabyn, HyGell, Col-Ace, Sure-Bet and Kelco Gel. The ice cream mixes were aged. The scores compared favorably with those of similar ice creams in which gelatin was used. In cases where a wheying off of the mix occurred, it was found that this separation could be prevented by reheating the mix after homogenization and cooling. This they take to indicate that the wheying off was caused by enzymatic action and that the enzyme was destroyed by the reheating of the mix in boiling water for a ten-minute

period. The use of superheated condensed milk in ice cream brings about an improvement in the body and texture. Its use, according to Sommer,[42a] is not as extensive as in the past.

During the course of this chapter frequent reference has been made to the use of gelatin and its effect upon the ice cream mix and of the body and texture of the finished product. These references indicate that gelatin raises the viscosity of the mix, interferes to some extent with whipping and improves the body and texture of the ice cream.

In an effort to learn more of the effect of gelatin in ice cream Leviton and Leighton [26] have measured the relative viscosities of ice cream samples in which gelatin content, fat content and homogenization temperature have been varied. They find much the same effects upon viscosity as when the fat content is varied. In other words an increase in the gelatin content from zero may first increase the viscosity of the ice cream slightly. Further quantities may then reduce the viscosity and still larger quantities again raise it. Under certain circumstances the decrease in viscosity is not obtained, in which case the viscosity rises slowly with increased gelatin content until a point is reached where the viscosity starts to rise at a greatly increased rate. In these experiments it was found that the most desirable ice cream was obtained when the quantity of gelatin was such that the viscosity was near minimum or at the point of breaking sharply upward. Increased fat content and increased homogenization temperature tended to reduce the amounts of gelatin necessary to produce given effects. Their conclusion is that in some way gelatin, homogenization temperature and fat are acting together to produce the same general effects upon the properties of ice cream.

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PART III

THE MICROBIOLOGY OF MILK AND MILK PRODUCTS

Chapter X

Sources and Distribution of Bacteria Found in Milk

Introduction

The subject of ecology is an important, but neglected, field of bacteriology. It is true that habitat has long been used in a superficial way in bacterial taxonomy, but serious studies of the interrelationships of bacteria and their natural environments have been sadly lacking. While no one would question the scientific interest of such knowledge, it is probable that its usefulness would be equally great. Our ignorance of the question, and the practical significance of such knowledge, are forcibly illustrated in connection with milk and dairy products. In treatises on systematic bacteriology we are familiar with such notations as, "habitat, milk"; but it would appear likely that the real habitat is to be sought in more fundamental sources. It is doubtful if many, if indeed any, of the "milk bacteria" are modified types whose present specific characteristics have arisen through an adaptation to milk, after production, as a natural habitat; more probable is it that these types existed in their present form in nature long before there was an organized dairy industry to supply them with a milk habitat. And, therefore, it is equally probable that such habitats are still the original sources of these organisms, however important such fortuitous objects as dirt, utensils, etc., may be as direct sources of contamination.

To illustrate the need of information of this nature it is only necessary to recall that the natural habitat of *Streptococcus lactis,* the premier milk organism first studied by Lister in 1878,[60] is as yet not definitely established. Still less is known of the probable original sources of a number of the important bacteria which function in the ripening of cheese. *Lactobacillus bulgaricus,* which has been extensively studied and widely used for practical purposes, comes from an unknown habitat.

Bacteria from the Environment

Bacteria from the soil. In a sense the soil may be looked upon as the most primitive and most important habitat of our economically important bacteria. On the other hand, most of the well-known species of hygienic and industrial importance appear to be types which have been modified through long adaptation to other environments, and their counterparts can not be found, at least in significant numbers, as natural soil inhabitants. This, in general, is true with respect to milk. While milk is

always contaminated with soil forms, and some of these types are present with sufficient constancy to be regarded as part of the normal milk flora, the more important of the common "milk bacteria" are derived from other sources.

But the soil is by no means an unimportant source of bacteria in milk. The constant occurrence of such soil types as *Bacillus subtilis* and other members of the aerobic spore-forming group in milk has long been recognized. Leaving aside the mooted question as to whether this group of bacteria is to be considered part of the actively growing and functioning soil flora, it is well known that they at least make extensive spasmodic growth in soil and occur there in large numbers, and hence the soil may be regarded as the primary source of such types in milk. Likewise, it is probable that the soil is the principal source of the anaerobic spore-forming bacteria which sometimes give rise to undesirable fermentations in milk and milk products. While it is true that one of the most active of this genus of objectionable bacteria in milk, *Clostridium welchii,* is more frequently of intestinal origin, the group as a whole is composed of types which are primarily soil forms, and it is probable that this is the source of a majority of the abnormal "butyric fermentations" and putrefactive taints which are not infrequently met in milk and milk products.

The so-called alkali-forming bacteria form another group of milk organisms which is primarily of soil origin. This term as applied to milk refers to non-proteolytic organisms which, when grown in milk, cause the development of an alkaline reaction. Specifically, this alkalinity results from a fermentation of the natural citric acid of the milk, and hence there would be grouped in this class all bacteria which can grow in milk, ferment citrates, and which do not cause an acid fermentation of lactose, or extensive proteolysis. Obviously this does not include a group of biologically closely related bacteria, but rather a miscellaneous assortment which may be conveniently considered together because of the similarity in their action upon milk. This grouping includes such diverse forms as *Alcaligines fecalis* and *Brucella abortus,* besides a great number of lesser known types. In their extensive study of the alkali-forming bacteria of milk Ayers, Johnson and Rupp [5] showed that the group is made up principally of non-spore-forming rods and a few cocci. They also showed that these forms are chiefly soil types, though the intestinal tract and udder were recognized as minor sources. That the soil should serve as the natural habitat of the alkali-forming bacteria of milk is most natural. Since the destruction of large quantities of organic matter in the soil is accompanied by the production of organic acids, it is obvious that bacteria which destroy these compounds which result from the primary fermentations of the carbonaceous substances in soil should play an important part in the decompositions which keep the chemical elements in constant rotation in nature.

Aside from the groups which have been discussed, the soil is undoubtedly the source of a number of other types of bacteria which sometimes make their presence felt in milk. Probably a number of the other

"abnormal fermentations" are caused by organisms derived from this source. The soil is known to be rich in thermophilic bacteria, and it is not unlikely that some of the thermophiles which occasionally cause trouble in milk have a favorable habitat for growth in the relatively hot surface layers of sun-exposed soil.

Water bacteria. Under modern conditions of milk production and handling, water is not usually an important source of bacterial contamination, though it must be recognized as a minor one. Certain types of bacteria which appear to be natural water forms, as the greenish pigment-forming organisms of the *Pseudomonas fluorescens* group, are also frequently found in milk. In fact, some of the earlier dairy bacteriologists interpreted the presence of such greenish colonies on milk plates as an indication of water pollution. As these bacteria are widely distributed in nature it is, therefore, a question if such an interpretation is justified. Water has long been looked upon as the source of bacteria which give rise to ropy or slimy fermentations which sometimes develop in milk. While it is not to be doubted that this has been the case in some instances, especially in the cases of polluted or stagnant waters, we do not have experimental data which would permit the conclusion that this is generally true.

Quite aside from the non-pathogenic, though obnoxious, bacteria which may get into milk from water supplies are the disease-producing organisms that are associated with polluted waters. The organisms of typhoid and paratyphoid fevers and, in other countries, Asiatic cholera and bacilliary dysentery, which are disseminated by sewage-polluted waters, grow well in milk and are frequently milk-borne.

Bacteria from plants. Plants as a source of bacteria offer an interesting problem and one which is of practical importance to the dairy industry. That the surface of plants may actually serve as a habitat for bacteria, as the skin of animals is known to serve for certain types, apparently has not been considered in a systematic way although numerous miscellaneous observations have been made. There can be no doubt that conditions on plants at least offer the opportunity for intermittent growth. Aside from the moisture derived from rain and dew it is known that plants exude considerable liquid which collects on the surface of the plant. Wilson,[124] who has studied this exudate, or water of guttation, showed that it exceeds in amount the commonly supposed values. Wilson has also shown that this exudate when collected makes an excellent culture medium for the growth of many bacteria. It appears to be accepted that some of the large ciliates among the protozoa exist and grow intermittently on moisture-laden plants; hence it would seem entirely reasonable that bacteria, whose generation time in reproduction is much less, might here find a favorable environment for growth. It is indeed possible that such terms as "the hay bacillus," "the potato bacillus," and "the grass bacillus," may have in some cases a more sound ecological basis than is ordinarily believed.

In addition to the possibility of furnishing a habitat for the growth of

bacteria, plants are important agents for the dissemination of the soil organisms with which their surfaces become contaminated. Of interest in this connection is the recent demonstration that anthrax cultures may constantly be recovered from the surface of plants grown in anthrax-infected soil. Although it is likely that the plant in such instances merely serves as a mechanical carrier, the possibilities of actual growth on its surface should not be entirely ignored.

Among the important milk bacteria one group has been shown to be present on plants and plant products in numbers which would suggest a definite ecological relationship, *Bacterium aerogenes* and its kindred species belonging to the aerogenes division of the so-called coli-aerogenes, or Escherichia-Aerobacter, group. Before a clear separation of the coli and aerogenes types had been established, Prescott [80] reported the finding of non-spore-forming aerobic, gas-forming, lactose-fermenting bacteria (then generally known as the "colon group") on plants and pointed out its significance in the interpretation of the results of bacterial tests on water supplies. Later Rogers, Clark, and Davis [89] discovered the fundamental difference between the coli types and the aerogenes types based upon the proportion of carbon dioxide to hydrogen produced by these organisms in the fermentation of glucose. The coli types were shown to produce the two gases in nearly equal amounts and in very constant ratio; approximately

$$\frac{CO_2}{H_2} = \frac{1.1}{1}$$

The aerogenes types, on the other hand, produced a larger proportion of carbon dioxide to hydrogen, the amount of carbon dioxide varying considerably, and a larger total amount of gas from the same amount of sugar fermented. The ratio of gases from the aerogenes types, the "high ratio group," ranged from

$$\frac{CO_2}{H_2} = \frac{1.5}{1} \text{ to } \frac{CO_2}{H_2} = \frac{3}{1}$$

Applying this knowledge to milk, Rogers, Clark and Evans [91] discovered a number of significant facts: Of a large collection of bacteria of the coli-aerogenes group from milk, approximately one-half were of the coli type while the rest belonged to the aerogenes division. Studies of cow feces [90] showed that nearly all of the organisms isolated from that source were of the coli type. An extensive investigation of human feces [92] yielded similar results. Grains were then studied and it was found that a vast majority of the group obtained from them were of the aerogenes type. On the basis of the investigations thus far at hand, the conclusion is reasonable that the coli-aerogenes organisms of milk, which are looked upon in some quarters as having a specific sanitary significance, are quite as apt to be of non-fecal as of fecal origin.

Plants as a source of organisms of the *Bacterium aerogenes* type are of especial importance in connection with "ropy" milk troubles. A large

proportion of the epidemics of ropy milk are caused by mucoid varieties of *Bacterium aerogenes,* or at least varieties of that group of organisms of which *Bacterium aerogenes* is the type species. Stark and his coworkers [109, 110, 111] have demonstrated the general presence in large numbers of mucoid types of *Bacterium aerogenes,* capable of producing ropy milk, on cattle feeds. In some cases these ropy milk organisms were present in ground grain dairy feeds in numbers exceeding 1,000,000 per gram. That these ropy strains should not necessarily be considered distinct species was shown by the fact that through dissociation non-mucoid strains, incapable of producing ropy milk, could be obtained from them. It was further shown that typical laboratory cultures of *Bacterium aerogenes,* which could not produce ropy milk, upon dissociation gave rise to mucoid types which produced abundant "ropiness" when inoculated into milk. This dissociation was brought about in different ways, but most readily through the action of a weak phage. The interesting fact that a *Bacterium aerogenes* phage, capable of inducing dissociation from the non-mucoid to the mucoid type, may be easily isolated from plants, was also shown by the Starks. Hence a plausible explanation of the natural occurrence of the mucoid type in large numbers on cattle feeds is suggested.

It is probable that some of the lactobacilli are associated with plants in nature. They are prominent in various fermenting vegetable products; for example, *Lactobacillus delbrücki* in grain mashes, *Lactobacillus pentoaceticus* in corn silage, and related types in sauerkraut and pickles. While these types are not ordinarily considered milk forms, it is possible that some of the milk types may come from vegetable sources. In fact, it has been shown [106] that organisms which are apparently identical with *Lactobacillus casei* of milk and cheese occur in rather large numbers on corn fodder.

The so-called aroma and flavor producing bacteria of butter starters, which are classified by different workers under the generic names of Streptococcus, Leuconostoc, and Betacoccus, are found on plant tissues and may have their habitat on plants. Organisms of this general group are prominent in fermentations of cabbage, beet, and other vegetable products, though there are not at present sufficient quantitative data concerning their occurrence on fresh plant tissues to establish a definite ecological relationship.[46, 57, 74]

Bacteria from dairy utensils. While dairy utensils are in no sense a natural habitat of bacteria, a great many organisms grow on the surfaces of such equipment so that the utensils form an important source of bacteria in milk. As a general rule, in fact, under ordinary farm conditions where steam is not available for sterilizing purposes, more bacteria gain access to milk through utensils than from any other source. It has been demonstrated by a number of workers that apparently clean but unsterilized utensils frequently add sufficient bacteria to increase the number in the contained milk several hundred thousand per cc. Indeed, under extreme conditions, unsterilized utensils may add more than one

million bacteria per cc. to the milk which is put in them.[4, 82] While it is true that under ordinary farm conditions the utensils are usually responsible for the largest number of bacteria of any single source of contamination, it should be recognized, on the other hand, that under good conditions, where proper methods of sterilization and drying of equipment are practiced, the utensils constitute only a minor source of milk contamination.

The subject of milk contamination from utensils is quite as interesting from the qualitative as from the quantitative point of view. Not only may dairy utensils add large numbers of microorganisms to milk, but among the forms which frequently get in from this source are many types which grow rapidly in milk and contribute to its spoilage. It was noted above that the natural habitat of *Streptococcus lactis* has not been established, but it is well known that the utensils act as an important, if not the most important, direct source of this organism in milk. Other important groups which have been found associated with dairy utensils are the coli-aerogenes types,[3] the alkali-formers,[5] miscellaneous non-spore-forming rods, and several species of micrococci.[85, 86, 119] Spore-forming bacteria are not as a rule prominent as contaminants from utensils, though it has been shown that they are sometimes added to milk in significant numbers from pasteurizing equipment, where the organisms appear to grow in the milk which has cooled on the surface of the pasteurizers.[87]

Bacteria from the air. Microorganisms which occur in air are of course in a dormant state. Quantitatively the atmosphere is a relatively insignificant source of contamination since the number of bacteria found in air is not large. Ruehle and Kulp[95] found that when the cows are in the barn, and during such operations as feeding and milking, ordinary stable air usually contains between 50 and 200 bacteria per liter, though results higher and lower than these were occasionally obtained. They further showed that when sterile water was "milked" in the stable through an apparatus designed to subject it to the same atmospheric contamination as is milk, the number of microorganisms thus added to the liquid averaged only 12 per cc. It is thus seen that under ordinary conditions of sanitation the stable air contributes an insignificant proportion of the bacterial count found in milk.

The types of bacteria which gain access to milk from the air are largely the types which are resistant to desiccation; namely, micrococci and the spore-forming rods. Mold spores are likewise prevalent in air, and it is possible that contamination from the atmosphere may assume practical importance in such products as condensed milk and butter, both of which are subject to commercial defects caused by mold growth.

Bacteria from the Milking Animal

Intestinal bacteria. The intestines of warm-blooded animals serve as the habitat of several types of microorganisms. Although a large variety of miscellaneous bacteria may be found in feces, there are only a

relatively few species which occur in very large numbers and hence appear to be quite adapted to the intestinal tract as a natural habitat. The selective environmental action of the intestinal canal is probably due in large part to the bile which is somewhat germicidal in its action toward many bacteria. In fact, there appears to be a restrictive action upon the growth of all of the intestinal bacteria while in the body of the animal. This is indicated by an immense increase in numbers of both fecal and non-fecal types of bacteria in the feces of man and animals after defecation.[3, 55, 81]

Cow manure has always been looked upon as the source of large numbers of bacteria in milk but experimental studies have shown that this source has been overestimated from the quantitative point of view. A simple calculation will readily show that the very high bacterial counts obtained from low grade market milks can not be explained to a significant degree by direct pollution with fresh cow manure. For example, the average bacterial content of fresh cow feces has been variously estimated at from 5,000,000 to 50,000,000 per gram (though individual samples vary from less than 1,000,000 to more than 500,000,000). If we accept the highest average figure it is found that the addition of 0.1 gram of manure to a pint of milk would increase the bacterial count only about 10,000 per cc. On the other hand, as was noted above, bacteria multiply rapidly in feces after it leaves the body, and this is also true of cow feces while it undergoes air drying. It is also true that milk is more frequently polluted with dry or partially dried manure than with fresh droppings. Studies of the bacterial content of fresh cow feces and the same samples after drying have shown[1, 81] that after drying two days at body temperature the samples usually contained over one billion bacteria per gram. On this basis, it is theoretically possible for 0.1 gram of the dry manure from the body of a cow to increase the germ content of a pint of milk more than 200,000 per cc. Notwithstanding the theoretical possibilities, the results of experimental studies have shown that under extremely dirty conditions the number of bacteria which get into milk from manure and other dirt from the body of the cow usually does not exceed 25,000 per cc.[4, 42] With average conditions of cleanliness the number so introduced is as a rule less than 5,000 per cc.; while under clean conditions the contamination from this source is in general less than 1,000 per cc. In fact, it has been demonstrated that milk can be produced under practical conditions with a bacterial content essentially the same as that found in the milk taken directly from the udders of the cows producing it.[104]

Qualitatively, the intestinal bacteria are very important, since from this source come a number of microorganisms which grow actively in milk and contribute to its spoilage. Some of them, notably members of the coli-aerogenes group and *Clostridium welchii* of the spore-forming anaerobic group, frequently cause gassy fermentations and undesirable flavors in milk and milk products. As was stated before, the members of the coli division of the coli-aerogenes group have their natural habitat in the intestinal tract and occur in large numbers, while the aerogenes types appear to be associated with plants. But the aerogenes types also

occur constantly in fecal matter, though in smaller numbers; whether these forms are also true inhabitants of the intestine, or whether they simply pass through the alimentary tract of the animal with its food, has not been determined. Other fecal bacteria which are of significance in connection with milk supplies are certain streptococci, especially *Streptococcus bovis* of the bovine mouth and intestinal tract, *Streptococcus fecalis,* and lactobacilli, as well as miscellaneous proteolytic types and alkali formers. From the standpoint of health bovine fecal matter must also be regarded as a potential source of the organisms of bovine tuberculosis. As has been shown by Schroeder [99] the tubercular cow swallows her sputum and large numbers of the organisms may pass unharmed through the alimentary canal and be discharged in the feces.

Bacteria from the skin of animals. The skin of animals serves as the habitat of a few bacteria, notably certain micrococci, but we have no experimental evidence to show that these natural skin microorganisms are of practical significance in milk, either from the quantitative or qualitative point of view. The coat of the milking animal is, however, of importance as a source of contamination with fecal and soil bacteria. The quantitative significance of this source of bacteria was experimentally demonstrated by Ayers, Cook and Clemmer [4] who included the proper care of the animal as one of the "four essential factors" in the production of milk of low bacterial content. Aside from intestinal, soil and natural skin types, the coat of the animal (because of the habits of the cow) may also serve as a means of contaminating the milk with bacteria from the mouth, especially certain types of streptococci and lactobacilli. In the case of diseased cattle we must also recognize this mode of contamination as a possible source of the organism of bovine tuberculosis.

Bacteria of the udder; Historical. In the early years of the science of bacteriology it was supposed that freshly drawn milk was sterile and would keep indefinitely if outside contamination could be prevented. This idea was based on the results of experiments reported in one of the original classics of bacteriology. In one of his experiments undertaken to show that all fermentative changes are dependent upon the growth of microorganisms, Lister [60] attempted to obtain milk without contamination. In his first experiment he prepared 12 test tubes with carefully drawn milk. They all developed growth, and Lister concluded that he had not used sufficient care in obtaining the samples. In the next experiment he put up 24 test tubes, using greater care, but they, also, all developed growth. "I felt little doubt that these organisms had got in for want of sufficient care on my part" he writes, and, undaunted, he prepared 12 more test tubes with extreme care, and in this third experiment was successful in obtaining two samples which remained sterile. Lister's interpretation of his results established the opinion of bacteriologists that milk within the udder is free from contamination, and it was many years before the true facts became acceptable.

The earliest recorded determinations of the bacterial content of aseptically drawn milk were made in 1891 by Schulz,[102] who reported that the

first milk to be drawn contained large numbers of bacteria and that the numbers decreased as the milking progressed. The results of Schulz and others who obtained similar results did not find ready acceptance, however, and the possibility of bacteria growing in the interior of the healthy udder was a moot question until the work of Moore,[68] and of Ward,[116] definitely established the fact that the normal udder may, and usually does, harbor bacteria throughout the whole extent of the lactiferous ducts, including the most minute ducts, where the milk becomes contaminated as soon as secreted.

Channels of udder infection. There are at least three channels through which bacteria may enter the udder. Disease germs which have invaded the blood stream may find lodgment in the udder and multiply there. They will be considered further on. Bacteria may be introduced into the udder by the penetration of sharp objects through the skin or by other injuries. The orifices of the teat canals serve as portals of entry for bacteria which can multiply in the small portion of milk remaining in the teat duct, and thus can work their way upward into the milk cistern and thence into the milk canals. The way is open, therefore, for all kinds of microorganisms for which milk is a suitable environment, but there is some kind of restraining action within the udder which controls the udder flora. The action is selective, so that only a few types of organisms are able to multiply in the udder and these never attain very great numbers, in comparison with the numbers of bacteria which occur in milk after it has been drawn and exposed to outside contamination.

The germicidal property of milk. There is in blood and in other body fluids a bactericidal power. Several kinds of "antibodies" are known to take part in the destruction of bacteria in the blood—agglutinins, precipitins, bacteriolysins and opsonins. Since the fluid part of milk, with many of its constituents, is derived from the blood, it is not surprising that the milk should be found to contain the same kinds of antibodies as the blood.

Fokker [36] was the first to call attention to the fact that there is a germicidal property in milk which may be demonstrated after the milk is drawn. Soon afterward Ehrlich [24] showed the presence of immune bodies in milk. Then Brieger and Ehrlich [18] demonstrated the presence of antitoxin in the milk of a goat immunized against tetanus toxin. Mice injected intraperitoneally with 0.2 cc. of milk were protected against 16 minimal fatal doses of toxin. Widal and Sicard [121] and others have demonstrated the presence of agglutinins in milk. Lane-Claypon [58] confirmed the work of other investigators in finding in milk both amboceptor and complement—the two factors concerned in cytolysis. They were found to be present in about one-tenth the amount of that in serum, varying slightly from day to day. Woodhead and Mitchell [126] have reported that opsonins may be demonstrated in milk.

The many workers who attempted to confirm or disprove Fokker's observation of the germicidal property of milk have not reported harmonious results. Rosenau and McCoy [93] called attention to the fact that

the agglutinins in milk were responsible for an apparent decrease in the number of bacteria in fresh milk. Due to the clumping of bacteria fewer colonies develop on the plates. These investigators agreed with the majority of others, however, that there is a restraining action in fresh milk.

The experimental evidence seems to have established that the germicidal property active within the udder exists to a slight degree after the milk is drawn. According to Heinemann,[45] this property survives 4 to 6 hours in milk kept at 37°, and in milk kept at lower temperatures it may survive as long as 24 hours.

There is a general agreement that the germicidal action of milk is so feeble, variable and transitory that it is of no practical significance in the preservation of milk. On the other hand, it may be an important factor in protecting suckling animals against infection. Famulener [33] carried out an extensive investigation on the transmission of hemolytic antibodies in goats. He concluded that such antibodies are transmitted from mother to offspring with the milk, and not through the placenta. This transmission of immunity was found to occur particularly through the colostrum during the first days of feeding. Smith and Little [108] noted the significance of colostrum in protecting the new-born calf against infection with intestinal bacteria.

Number of bacteria in the udder. It would be impractical to attempt to quote all the numerous investigators who have studied the flora of freshly drawn milk to determine the number of bacteria which may exist within the udder. Gorini [40] found that the number varied from zero to 300,000 per cc. Hastings and Hoffman [44] studied the bacterial content of milk from the udders of 3 cows and found that their averages varied considerably. These investigators found that the same organisms may persist in the udder of the cow for long periods. They recommended that in sanitary dairies the normal content of the udders of the individual animals may well be taken into account. Harding and Wilson [41] studied 900 samples of milk and found an average content of about 500 bacteria per cc. They found that there may be a considerable difference between the numbers of bacteria in any two quarters of the same udder. There is general agreement with these results which have been quoted in regard to the number of bacteria in freshly drawn milk. Steck [112] found that the bacterial species present in the four quarters of the udder are rarely the same, but that there is a constancy shown both qualitatively and quantitatively in the milk from single quarters, in that the same species were excreted in approximately constant numbers from month to month, or even from year to year. Sherman [104] states that by a selection of cows according to the known bacterial content of their fresh milk, marked differences in the bacterial content of the mixed milk could be obtained.

It was noted above that the types of bacteria which can grow within the udder are limited. There are only 4 general groups of organisms that commonly grow in healthy udders—staphylococci, streptococci, diphtheroids, and minute Gram-negative rod forms of which the organism of

contagious abortion is the type species. *Brucella* is the commonly accepted generic name for the latter group, although they are classified by Bergey [11] under the generic name *Alcaligines.*

Staphylococci. Gorini noted that staphylococci were the most common bacteria in aseptically drawn milk, and soon afterward von Freudenreich and Thöni [113] found that liquefying "micrococci" were the predominating organisms in milk drawn with precautions against contamination. Evans [25] found staphylococci in 58.8 per cent of 192 samples of milk aseptically drawn from single quarters of normal udders. The numbers varied from a very few to 80,000 per cc.

Of 35 cases of bovine mastitis studied by Savage [97] 16 per cent were found to be due to staphylococci. Jones [53] found staphylococci associated with bovine mastitis in 24 out of 81 cases. Evans obtained staphylococci highly virulent for rabbits from aseptically drawn milk taken from a herd which was supplying certified milk.

In a search through the literature, only two reports of human infections with bovine staphylococci were found. Barber [7] reported that repeated attacks of gastroenteritis occurred among residents and visitors at a certain farm in the Philippine Islands during a period of three years. Barber found that the illness was due to a toxin elaborated by a white staphylococcus, which occurred in the udder of a cow. The cow was apparently in good health during this time, except for one attack of garget.

Ramsey and Tracy [88] reported that one of them was afflicted with a severe gastroenteritis while studying the production of an undesirable flavor in a commercial plant. A suspicion that the disease was due to drinking a milk culture of an orange-colored *Staphylococcus aureus* from the udder, an organism which attacked casein with the formation of a malt-like flavor and odor, was confirmed by feeding some of the culture to kittens, which developed diarrhea.

Although there is scant evidence that udder staphylococci are pathogenic for man, the possibility should not be overlooked. Jordan [56] cites a number of instances in which food poisoning was due to staphylococci of undetermined origin and concludes that *Staphylococcus aureus* (and *albus*) must henceforth be ranked among the bacteria of known toxigenic power.

Streptococci. Although staphylococci are the bacteria most commonly found in aseptically drawn milk, their importance is secondary to the streptococci, which demand more interest on account of their relationship to the health of the cow and to human health.

Streptococci occur very frequently in the udders of normal cows. Sherman and Hastings [105] found streptococci in 0.01 cc. of the mixed milk from 10 out of 12 herds, and in 38.6 per cent of the individual samples from 88 cows of 4 herds. One of the herds tested was well known to the authors, and so far as could be learned, there had been no case of udder inflammation and no trouble had resulted from the use of this milk which was sold largely for the feeding of children. Evans [25] obtained

streptococci from 15.1 per cent of 192 samples from the single quarters of the udders of 161 cows of 5 different dairies. The highest number found per cc. was 264,000. Ayers and Mudge [6] obtained streptococci from the milk directly from the udder of 38 per cent of 133 normal cows examined.

The observation of Rogers and Dahlberg [88] that udder streptococci may be differentiated from the common milk-souring organism, *Streptococcus lactis* (Lister), by their behavior in litmus milk, was utilized by all of the quoted investigators of udder streptococci. *Streptococcus lactis* decolorizes litmus milk promptly, leaving a white curd with a pink ring at the top which slowly extends downward. Udder streptococci usually curdle the milk, and reduction of litmus may take place after curdling, but the dye is never completely reduced. The authors quoted all agree that *Streptococcus lactis* does not multiply in the udder. Earlier reports to the contrary were probably due to a failure to distinguish this organism from the bovine type of udder streptococcus.

Streptococcus mastitidis (Guillebeau), the common udder streptococcus is characterized by Ayers and Mudge as follows: "It does not reduce methylene blue; coagulates litmus milk usually in 24 hours, and partially decolorizes the milk after coagulation; does not grow in milk at 10°. It ferments dextrose, lactose, and sucrose and may or may not ferment salicin. It does not ferment mannite, raffinose, or inulin. It produces CO_2 and NH_3 from peptone, but no CO_2 from dextrose. It hydrolyzes sodium hippurate into benzoic acid and glycocoll. There are two varieties; one which produces the beta type of reaction, and the other which produces the gamma type on blood agar plates."

Ayers and Mudge found that 79 per cent of their strains of udder streptococci were *Streptococcus mastitidis*—64 per cent belonging to the beta variety which gives a zone of clear hemolysis on blood agar plates and 15 per cent belonging to the gamma variety, which gives no hemolysis or only slight greenish color around the colonies. These investigators found the same types of streptococci in normal udders as in the cows with mastitis, the only difference being the larger numbers found in cases in which mastitis was present.

Jones [52] also regards the streptococci from apparently normal udders as identical with streptococci which commonly cause mastitis. He groups the carriers as follows: (a) those that have been infected recently and have not yet developed symptoms; (b) those that have suffered from inflammation of the udder and after recovery still harbor streptococci; (c) those that never show symptoms of mastitis.

Jones examined the milk from 85 cows suffering from various forms of mastitis and found non-hemolytic streptococci in 35 cases and hemolytic streptococci in 17 cases. In two instances both types were present. Savage [97] found that the majority of cases of acute mastitis were due to *Streptococcus mastitidis.*

Evidence has never been brought forth to show that the streptococci which are the usual cause of mastitis in cows are of any significance to

human health. It appears that the bovine type of streptococcus is generally avirulent for other species of animals. Nocard and Mollereau [72] were the first to investigate this subject. They fed young dogs and young rabbits with cultures of streptococci obtained from cases of mastitis. No ill effects were noted and they concluded that milk containing these organisms could be used for food without danger. Jones found that freshly isolated strains of the non-hemolytic type of bovine streptococcus failed to produce marked effects when inoculated into rabbits. He fed a young pig with about two quarts of purulent milk a day for 15 days. The pig was under observation for 10 days after the feeding was discontinued but failed to show symptoms of any disorder. Savage found the bovine type of streptococcus of low virulence for laboratory animals, but capable of causing mastitis in goats. This investigator was so confident that the bovine type of streptococcus was incapable of causing human disease that he introduced into his own throat massive doses of a strain obtained only a day or two previously from a case of bovine mastitis. He suffered no ill effects from the experiment.

Although the common causal organism of mastitis in cows is harmless to man, there is another type of hemolytic streptococcus, pathogenic for man, which does occasionally infect the udders of cows. A number of severe epidemics of sore throat have been caused by the consumption of raw or imperfectly pasteurized milk from animals infected with this organism, generally known as *Streptococcus pyogenes,* or in recent years commonly called *Streptococcus epidemicus.*

According to Ayers and Mudge, *Streptococcus pyogenes* may be differentiated from *Streptococcus mastitidis* by several tests. When both types are grown in dextrose broth, the hydrogen-ion concentration produced by the bovine type is at about pH 4.5, whereas the final value produced by the human type is at about pH 5.5. The bovine type hydrolyzes sodium hippurate but the human type does not. The human type is about 100 times more hemolytic than the bovine type, when hemolysis is measured in tubes. The two types may also be differentiated by serologic tests.

There has been a considerable amount of evidence collected which indicates that cows infected with *Streptococcus pyogenes* receive their infection from human sources. Capps and Miller [17] and Davis [20] were the first to present data bearing on this problem. They traced the causal agent of the Chicago epidemic to a certain dairy in which 4.6 per cent of the cows were suffering from mastitis. It was found that sore throat of the epidemic type was prevalent among the farmers and milkers supplying the dairy. A streptococcus was obtained from a typical case of mastitis in a cow on one of the farms, and a streptococcus identical with the bovine strain in morphology, in culture, and in pathogenicity was obtained from a human case of tonsillitis and arthritis on the same farm. Later Davis and Capps [21] produced mastitis experimentally in cows by inoculating the udder with a strain of streptococcus recently obtained from a human case of tonsillitis. The disease in the cows lasted for several weeks, corre-

sponding roughly to the duration of the milk-borne epidemics. The disease sometimes showed no physical evidence of its existence, although large quantities of pus and streptococci were being excreted in the milk. The observations of Capps and Miller and of Davis have been confirmed by other investigators of later epidemics.[9, 14, 94] A good review of the subject is given by White[118] in connection with the Lee epidemic of septic sore throat reported by Bigelow and White.[12]

Brucella. In 1911 Schroeder and Cotton[100] reported that they had found the organism of infectious abortion in 8 of 77 samples of market milk tested (over 10 per cent) and in the milk distributed by 6 of 31 dairies (over 19 per cent). Schroeder[101] afterwards reported a higher percentage of milk samples infected with this organism. He injected into guinea-pigs 516 samples of milk from 90 dairies, and 103 of the animals developed the characteristic disease. The results showed that the milk from 29 of the dairies was infected from time to time with the abortion organism.

The findings of Schroeder and Cotton were confirmed by other investigators in other sections of the United States. Fabyan[32] working in Massachusetts examined the milk from 12 cows of a pure bred Guernsey herd. He found the abortion organism in two of the samples. Huddleson[47] noted its prevalence in cow's milk in Michigan, and Fleischner and Meyer[34] noted its presence in California. That the organism of contagious abortion is present in cow's milk in Europe was established by the findings of Zwick and Krage[127] and of Winkler[125] and others. The results of these early investigators have been amplified by an ever increasing volume of literature which shows that in every part of the world wherever cattle are kept, they are subject to infection with Brucella. Contagious abortion is a problem in the East Indies, South America, South Africa and Australia, as it is in the United States and in Europe. Poppe[78] gives a bibliography of the literature on the worldwide distribution of the cattle disease.

The significance of the organism of contagious abortion to human health became a problem of great interest when it became known that it is so closely related to the organism which is common in goat's milk and causes Malta fever in man that strains from the two sources can not be differentiated by ordinary laboratory methods. This observation was first made by Evans,[27] and was confirmed by Meyer and Shaw[65] and later by numerous other investigators. The bovine and caprine types of *Brucella* are now considered varieties of the same species—*melitensis.*

In 1886 Bruce discovered the causal organism of Malta fever, and a couple of decades later the British Royal Commission established that the chief source of human infection is goat's milk. It was about forty years after Bruce's discovery that the human disease due to *abortus* infection was recognized. The first case was diagnosed in 1922, and was reported the following year. For the next three or four years only a few cases in gradually increasing numbers were recognized in several centers where research in this disease was being conducted. In 1927 the phy-

sicians of this country began to be aware that undulant fever should be considered in every case of fever of undetermined origin, and 217 cases were reported. As the information was more widely disseminated there was a rapid increase in reported cases, until now over 1,500 cases are reported annually.

The reported cases of undulant fever are those characterized by pronounced symptoms. The disease is known to exist also in a mild chronic form which is extremely difficult to diagnose. Undoubtedly there are great numbers of unrecognized cases of this mild form of the disease.

It is impossible to ascertain to what extent the increasing number of reported human cases of undulant fever is due to an actual increase in cases. The increased sharpness in diagnosis and alertness to the danger of infection would account for growing numbers of reported cases. However, according to Mohler,[67] Brucella infection of domestic animals is known to have become more widespread in this country in recent years, until it now causes greater losses than any other animal plague. Probably there is a corresponding increase in the number of human cases of infection.

Brucella organisms introduced through the skin are more infective than those consumed in food. Consequently undulant fever, or brucelliasis, as the disease is now sometimes called, is largely an occupational disease among farmers and veterinarians who handle aborting animals, and among slaughter house workmen who handle infected carcasses. According to the data of Hardy and his colleagues [48] 62.5 per cent of cases occurring in Iowa were among those who had contact with live stock or carcasses.

There are many cases of the disease recorded in literature in which there had been no contact with infected animals, but in which raw milk known to be infected had been consumed. A group of such cases was thoroughly investigated and reported by Atwood and Hasseltine.[2] They studied an epidemic of 11 cases in a town of 23,000 inhabitants, and traced the source of infection to raw milk.

Although the *abortus* variety of *Brucella melitensis* is commonly present in cow's milk, it has never been shown to exist there in great numbers. Most of the investigators who have demonstrated it in cow's milk have done so by inoculating the milk into guinea-pigs. The guinea-pigs are highly susceptible to infection and develop lesions from which the organism may be obtained. Huddleson [47] perfected a method for cultivating *abortus* directly from milk. Later Giltner, Huddleson and Tweed [88] expressed the opinion that the organism occurs in cow's milk only in very limited numbers. They reported that the organisms in a single 10 cc. sample would vary from 1 to 200. Many years previously Shaw,[103] studying *melitensis* in goats and other animals in Malta, found that it occurred in cow's milk only in limited numbers. His results were comparable with the numbers found by Giltner, Huddleson and Tweed. He supposed that he obtained the true *melitensis* from cow's milk. But

there is no way to know, now, whether he cultivated the *melitensis* or the *abortus* variety.

In striking contrast to the limited numbers of pathogenic *Brucella* in cow's milk are the very great numbers in which it may occur in goat's milk. Eyre, McNaught, Kennedy and Zammit [31] frequently found 30,000 per cc. It appears that by their method of plating higher numbers could not be developed. They remark that "on several occasions control plates were prepared with suitable dilutions of milk and it was frequently found that 3,000,000 per cc. would be much nearer the true content of the milk than the 30,000 recorded."

In the case of both the cow and the goat, *Brucella* are commonly excreted from apparently normal udders of healthy animals. Schroeder and Cotton [100] found at necropsy nothing except a few small areas of slight induration in the udder to explain the persistent occurrence of the organism in the milk of the cows that were studied. Runnells and Huddleson [96] examined the infected udders of 3 cows in which there was no gross evidence of disease. They found numerous small inflammatory foci varying in degree from acute and subacute to chronic. In goats, as in cows, the chief symptom of infection is the premature expulsion of the fetus. According to Dubois [23] goats may sometimes show other symptoms. There may be lactation troubles consisting of an alteration of the milk which "turns" as soon as drawn. Quite often in the course of the disease goats develop a subacute bronchitis, with a cough of variable intensity. Burnet and Anderson [15] have reported that lesions may be found frequently in the mammary gland of infected goats with definite abscess formation in some cases. The more severe type of the disease in the goat appears to be correlated with the greater numbers of the organism excreted in goat's milk. The great numbers of *Brucella* in goat's milk, as compared with the few in cow's milk, must be one of the factors which makes human infection with goat's milk more common than with cow's milk. Further, the variety of *Brucella* which commonly infects goats is more virulent for man than the variety which commonly infects cattle.

Evans [30] gives the following general description of *melitensis,* the type species of the genus Brucella:

> "Minute rods with many coccoid cells (the cells of 2-day cultures grown on the surface of plain agar and stained with carbol fuchsin appear about 0.5 of a micron wide and 0.5 to 2 microns long); not forming endospores; non-motile; aerobic, or preferring a slightly reduced, partial pressure of oxygen; without gelatin liquefaction; gram-negative; parasitic, invading animal tissues; neither gas nor acid production from the carbohydrates." Evans [30] distinguished eight serologic groups of *Brucella melitensis* by the agglutinin absorption test. Only two of these serological groups are common in the United States—the bovine type, which is designated variety *abortus,* and a type designated *melitensis* A which is common in goat's milk in the southwestern states, and was also found to be distributed sporadically in other parts of the United States.

The *abortus* variety, as determined by agglutin absorption, has been further divided by Huddleson [48] into two groups, according to response to the bacteriostatic action of certain dyes.

The porcine type of Brucella, designated *suis,* resembles the caprine type in being more virulent than the bovine or *abortus* variety. (Huddleson and Hallman.[49]) The *suis* variety, however, may infect cattle. The milk from the infected udders is then much more dangerous to human health than milk containing the common or *abortus* variety of the organism.

The complete eradication of undulant fever from the British garrison in Malta, following the discovery that goat's milk was the chief source of human infection, was a dramatic event in medical history. The possibility of undulant fever infection from cow's milk being recognized, the precautionary measures were obvious. The command was given forbidding the soldiers to drink raw milk, and the result was prompt and unmistakable disappearance of the infection. Unless milk is known to have come from an abortion-free herd, it should be pasteurized.

Evans [28] described a number of *abortus*-like bacteria from the udder which agglutinate only in low dilutions of *abortus* serum and differ slightly from the pathogenic type in biochemical reactions. They were found more commonly in milk from cows which had aborted than in milk from cows which had not aborted.

Diphtheroids. Not much attention has been paid to the diphtheroids in aseptically drawn milk, presumably because special methods of culturing are required to reveal them. The first mention of them seems to have been by Bergey,[10] under the name of *Bacillus pseudodiphtheria.* Bergey, however, did not describe them. Fourteen years later Evans [26] described them under the name of *Bacillus abortus* variety *lipolyticus,* the variety name having been suggested by the most conspicuous biochemical property of the organism. In a later publication [27] a further description was given under the name *Bacterium lipolyticum.* The characteristics of this organism agree with those cultivated by Preisz [79] from the uterine secretions following abortion and described under the name *Corynebacterium abortus endemici.* This organism is harmless when injected into experimental animals, as shown by Preisz and also by Evans. It also differs from the pathogenic *Brucella* culturally and in staining reactions, although growth in agar shake culture resembles that of the causal organism of contagious abortion, according to the early description of Nowak.[78] Evans found it in a higher percentage of samples of milk of cows which had aborted, than in the milk of cows which had not aborted. In a recent publication Bendixen [8] reported from Denmark, on the contrary, that he found them as frequently in the milk of cows which had not aborted as in that of those which had aborted. Ritter [84] found little or no evidence of reaction in the udders of cows yielding milk containing diphtheroids.

Diphtheroids may occur in great numbers in aseptically drawn milk. Evans [26] found them in 73.9 per cent of samples of milk from one dairy, with 112,000 per cc. in the most heavily infected sample. Steck [112] confirmed the presence of these organisms in aseptically drawn milk. Using a special medium Dorner [22] found them to be the dominant species in aseptically drawn milk from 108 cows of several herds in this country. He

found them in 34.8 per cent of samples of milk, with an average count of 9,700 per cc. Dorner examined aseptically drawn milk in Switzerland by the same method, and there found the diphtheroids to be less frequent than micrococci.

According to the description given by Evans the diphtheroids of milk are characterized particularly by their growth in agar shake culture in a layer a few millimeters beneath the surface, and by their ability to hydrolyze butter fat. In litmus whole milk there is a rapid development of the cells, and a slow development of acid, which is first evident in the cream layer. After a couple of weeks' incubation at 37°, the optimal temperature, the cream is converted into a firm, slightly granular, ill-smelling mass. On agar slope the growth is scant, in discrete colonies. The addition of bile or blood serum to the agar promotes a more vigorous growth. On agar containing blood serum the diphtheroids produce a brownish discoloration. They produce no effect on gelatin, nitrates, urea or asparagin; they produce no acid from the carbohydrates and related substances which are commonly used for fermentation tests. In broth the growth is scant in a granular sediment with clear medium above.

Besides the four groups of bacteria,—staphylococci, streptococci, Brucella and diphtheroids,—which commonly exist in cows' udders, organisms of entirely different nature sometimes become established there. These peculiar infections are liable to run through many cows of a herd. Moore and Ward [69] found that the udders of many cows of a certain dairy were infected with a gas-producing organism resembling, if not identical with, *Bacillus coli communis.* This organism was responsible for the production of peculiar taints in the milk and cheese and gassy curd. Evans [29] found *Actinomyces* (*Streptothrix*) in the milk from 18 out of 21 cows of one herd. The udders of these cows did not appear to be abnormal.

Both Jones [51] and Savage [97] report that they have found *B. coli* associated with mastitis in cows. According to Wall [115] *Actinomyces* (*Actinomyces farcinicus Nocard*) may cause mastitis in the cow. Another organism which may cause mastitis in the cow is commonly known as the "pyogenes bacillus" (*Actinomyces necrophorus Löffler*). So far as known, this organism is not pathogenic for man.

A review of the flora of normal and diseased udders brings out the fact that the pathogenic organisms which cause mastitis in cows are closely related to organisms which may exist in the apparently normal udders. This is true of the more unusual infections, such as *Escherichia coli* and *Actinomyces,* as well as in the case of the organisms which commonly cause infection—*Staphylococcus, Streptococcus,* and *Brucella.*

Anthrax. Three important general diseases of cattle—anthrax, foot and mouth disease, and tuberculosis—deserve consideration here because the etiologic agents are pathogenic for man. Of these, anthrax may be dismissed briefly, for, according to Hutyra and Marek [50] the anthrax bacillus does not pass from the blood into the milk until the last hours of life. Moreover, the milk secretion ceases almost completely very early

in the disease. If there is a slight secretion of milk it is yellowish or bloody in appearance and slimy. The merest grain of common sense would exclude such milk from use.

Foot and mouth disease. In the world at large, foot and mouth disease is one of the most important of the infectious diseases of cattle. In the United States the disease has been limited to a small number of outbreaks, all of which were eradicated by quarantine and slaughter of the exposed or infected animals. The carcasses of such animals are either buried deeply after treatment with quicklime, or destroyed by burning. The disease is caused by a filterable virus, the nature of which is little understood. According to Mohler [66] the virus is present in the serum of the vesicles on the mouth, feet, and udder; in the saliva, milk and various other secretions and excretions; also in the blood during the rise of temperature. The udders often become inflamed and ruined by the formation of abscesses, and cows affected in this way may be rendered permanently valueless for milk production. The milk obtained from cows suffering from foot and mouth disease is diminished in quantity and is not readily converted into either butter or cheese, but remains thick, slimy, and inert in spite of churning and attempts at curdling. Such milk is dangerous for use, causing fatal diarrhea in suckling calves or young pigs and serious illness in human consumers. It has been found that the milk may be infected even before the formation of the blisters in the mouth, on the hoofs, or in the udder, has taken place.[114] The disease usually develops in a mild form in human adults. Serious infections in adults have occurred, however, during some European outbreaks. In children the disease is more often severe, and an associated gastro-intestinal catarrh may lead to death.

Tuberculosis. On account of its wide distribution, chronic nature, and its important economic and sanitary significance, bovine tuberculosis has received more attention in the United States than any other cattle disease. The early bacteriologists considered the causal organism of the human disease identical with that of the bovine disease. Theobald Smith [107] was the first to raise the question of the duality of human and bovine tuberculosis. Then, in 1901, Koch announced to the International Congress on Tuberculosis held in London that in his opinion the bovine type was not pathogenic for man. Immediately following this convention commissions to investigate this problem were appointed in the United States, England, France, and Germany. From these investigations it was learned that the bovine strains belong to a distinct type which differs from the human type in several characteristics, including morphologic, cultural, and pathogenic differences.

The bovine strains tend to be shorter and thicker, and stain more uniformly than the human strains. In the first few generations on culture medium, strains of the bovine type give much less vigorous growth than those of the human type. With continued cultivation, however, the bovine strains show increased vigor of growth until they become indistinguishable from the human type. Strains of the bovine type possess a higher degree

of virulence for mammals, excepting man but including the ape, than strains of the human type.

There appears to be general agreement that the human and bovine types of the organism of tuberculosis (*Mycobacterium tuberculosis*) are races of the same origin which have acquired their characteristics by passage through one or the other species. The real problem concerning the identity or duality of the types is how readily the one may be converted into the other. Calmette [16] has reviewed the literature on this subject and concluded that the question of experimental transformation of the human type of the organism into the bovine type can not yet be regarded as settled; and that there has been no greater success in artificially transforming the bovine type into the human type.

Nevertheless, it was long ago established that the bovine type of organism may cause tuberculosis in man. Park and Krumwiede [77] studied a large number of cases and concluded that infection of adults with the bovine type of the organism is uncommon. Although cases of pulmonary tuberculosis due to the bovine type of the bacillus have been reported, such cases are rare. The data show, however, that 20 per cent of abdominal tuberculosis and 23 per cent of tuberculosis of the skin are due to the bovine type of the organism. In children the majority of the cases of tuberculosis of the skin and tuberculosis of alimentary origin are due to the bovine organism. These investigators found that from 6 to 10 per cent of all deaths caused by tuberculosis in children under five were due to bovine infection. After a review of the literature Calmette pointed out that the frequency of human infection with the bovine type of the organism varies in different countries, and that in Paris it is much less than in London and in New York.

The prevalence of tuberculosis in cattle is therefore a problem of great importance. The average proportion of tuberculous cattle in the United States from 1893 to 1908 as determined by the tuberculin test was 9.25 per cent.[64] In certain localities much higher percentages of the cattle have been found infected. The tuberculin test revealed infection in many apparently healthy cattle.

According to Wall [115] about 3.5 per cent of tuberculous cows are affected with udder tuberculosis. Infection from a lung lesion through the blood stream is the most common method of transmission to the udder, though infection through the teat canal may also occur. In tuberculosis of the udder, the milk is always infected, and sometimes great numbers of the organism may be demonstrated. Ostermann [75] found that 1 cc. of milk diluted 1 to 50,000 injected intraperitoneally into a guinea-pig would produce fatal tuberculosis. Furthermore, animals with even a mild infection may at any time suffer an attack in the udder through metastasis, and neither the beginning of the attack nor the stage of the disease can be recognized by a clinical examination. Hence the milk from infected cows may contain the tuberculosis organism even though the udder is apparently healthy. Recent experiments have shown that cows in

apparently perfect health which react to the tuberculin test may discharge tubercle bacilli with the milk.

Raw milk from tuberculous cows is not only dangerous for children, but it also transmits the disease to calves, swine, or other animals which may be fed with it. Feeding separated milk from creameries and whey from cheese factories has in the past been an important means of spreading the disease.

Though tuberculosis among sheep and goats is of infrequent occurrence, cases of infection probably result from exposure to the bovine type of organism. The disease in these animals is similar to that in cattle, and the mammary gland may be infected in the same manner in all three species.

Milk-Borne Epidemics

General discussion. The danger of human infection from milk or its products is unfortunately not limited to milk from infected animals or to diseases caused by organisms infectious for cattle. Milk is an excellent culture medium for many bacteria pathogenic to man, and, even when it is held at a temperature low enough to retard or inhibit bacterial multiplication, such organisms may remain viable and virulent for long periods. For example *Eberthella typhi* has been known to persist for weeks in ice cream. Typhoid fever, septic sore throat, undulant fever, scarlet fever, diphtheria and the intestinal infections of childhood are often spread by milk, and more rarely paratyphoid, dysentery and other diseases. Any organism capable of surviving several hours in milk, and infectious if introduced into the mouth, can be so conveyed.

Bacteria pathogenic to man get into milk in a variety of ways. A milker or a handler of milk may be suffering from a disease in a mild form, or he may be healthy but a carrier, particularly in the case of typhoid fever or diphtheria. Utensils may be contaminated by being washed or rinsed in polluted water, or by being handled by a person in contact with a patient in the farm house. Rats, mice, flies and domestic animals, if allowed access to utensils or to milk, may carry infectious material to it. Organisms thus introduced are soon dispersed through the liquid, and when large amounts are mixed together and then distributed, the number of people that can be infected from a single source is multiplied. When a large city milk supply is thus invaded a severe epidemic may result.

A disease is said to be epidemic when its incidence in a community rises above normal expectancy. In any large group of people there will always be a few cases of the ordinary infectious diseases and it is well known that most of them exhibit a periodic variation in numbers, caused by climatic conditions, or by the circumstances of civilized life. For example, diphtheria usually shows a distinct rise in the autumn after the children are reassembled in school, but such a rise is not considered to constitute a true epidemic.

General characteristics of milk-borne epidemics. Milk-borne epi-

demics have certain recognizable characteristics. The onset is usually explosive; that is, persons in considerable numbers are exposed at the same time, and consequently develop their initial symptoms within a period of a few hours or days, the variation depending on the amounts of infectious milk ingested and the individual resistances of the persons attacked. When many people sicken simultaneously, the milk or water supply is to be suspected, if the disease can be so spread. Explosive outbreaks can, however, be caused by direct contact or droplet infection, when large numbers of children or adults are assembled in close proximity, as in boarding schools, military camps, or institutions. Epidemics have been caused by foods other than milk, but more rarely, as it is not often that a very large number of persons partake of the same batch of food.

Milk-borne epidemics may frequently be recognized by a selective incidence limited to milk drinkers. There are often more cases among women and children than among men, and more among the prosperous than among the poor. Neighborhoods where the sanitary conditions are excellent do not escape, as they often do in epidemics spread by other means. Furthermore, a milk-borne disease will break out simultaneously among people who have no social contacts with one another, and whose children attend different schools; for the milkman makes neighbors of families living miles apart. Some city health departments make a practice of charging each case of typhoid, diphtheria, or scarlet fever against the dairyman supplying the household and thus occasionally recognize a milk-borne epidemic earlier than would otherwise be possible, by the abnormal number of cases among the patrons of a particular supply.

As children drink more than adults, an epidemic showing a higher percentage of children suggests milk as a possible cause. However, in the case of children's diseases to which adults are relatively not susceptible, an early age incidence means little if anything. The fact that an outbreak of diphtheria is almost entirely confined to children does not by any means incriminate the milk supply, unless other evidence points in that direction. On the other hand, typhoid fever is usually contracted between the ages of 15 and 30, and a typhoid epidemic with many cases under 15 points to milk as the probable conveyor of the disease.

Occasionally there are small outbreaks due to bottles returned from the home of a patient and used again without sufficient sterilization. As only a few bottles are thus contaminated each day there will be only a few cases developing at any one time, and such a state of affairs is not easily recognized as caused by milk. Bottle-borne infection may be a complicating side issue in a typical milk-borne epidemic, when bottles from the contaminated source are turned in to other dealers.

Once the source of infection has been located, and the offending milk withdrawn from the market or rendered free from contamination, a typical milk-borne epidemic ceases almost as suddenly as it began, although there are sometimes secondary cases among individuals in contact with those who were infected through the milk.

In recent years there have been more milk-borne epidemics of typhoid

fever than of any other disease, but septic sore throat and scarlet fever, which come respectively second and third in number of epidemics, have caused more cases of illness. Within the past decade very important advances have been made in our knowledge of these two diseases. The long-debated question as to the etiology of scarlet fever has been settled to the satisfaction of most medical bacteriologists, and it has become increasingly evident that the streptococci of scarlet fever are very closely related to those causing epidemic sore throat.

Epidemic sore throat. This infection is characteristically spread by milk, with an almost negligible number of secondary cases apparently due to contact. It is in no sense a children's disease, the mortality being greatest in the late twenties. Some of the epidemics have been extremely severe, notably the 1911 outbreak in and near Boston, with over 1000 cases, and one in Chicago the following year estimated at 10,000. Epidemics have occurred in England and on the continent of Europe.

Hemolytic streptococci have been isolated from the patients' throats and from the milk with such regularity that it is generally conceded that they are the cause of the disease. In the years since the first disastrous outbreaks, methods of differentiating human from bovine hemolytic streptococci have been discovered and developed, and, as stated earlier in this chapter, it is now the generally accepted opinion that the septic sore throat streptococci are of human origin, but are capable of establishing themselves within a cow's udder and multiplying there, in some cases without producing noticeable inflammation. The epidemics usually cease with remarkable abruptness as soon as the source is located and the milk withheld from circulation.

Scarlet fever. The majority of scarlet fever epidemics are not traceable to the milk supply, but every year there are a few so spread. The source of contamination may be an unrecognized case among the milkers or dairy employees from whose mouths and noses the streptococci gain access to the milk. Jones [54] has suggested that in these outbreaks, as in those of septic sore throat, it is easier to explain the large numbers of streptococci present in the milk by the existence of a focus of infection in the cow's udder than by direct human contamination. His experiments with cultures of scarlet fever streptococci indicated that normal milk from an uninfected udder usually has sufficient bactericidal power to inhibit the multiplication of the relatively small numbers that would be introduced by sneezing or coughing. The similarity between the sore throat organisms and the scarlet fever ones does not end here. In both cases they are of the β or hemolytic type and in neither case is a single agglutinative type found. The exotoxin which causes the rash and desquamation characteristic of typical scarlet fever is not confined to a single agglutinative or biochemical type, and strains vary in their toxicity as well as their other pathogenic properties. Moreover, human beings vary considerably in their susceptibility to this exotoxin and in their resistance to the invasiveness of the cocci themselves. Consequently, a typical scarlet fever strain, in a given individual, may fail to produce a rash or noticeable

fever, and the case may not be diagnosed. On the other hand, if this individual, resistant to the action of the exotoxin, is nevertheless still susceptible to the other "endotoxic" activities of the organism, the result may be indistinguishable from typical septic sore throat. This is well illustrated in the epidemic reported by Welch and Mickle.[117] Of these 100 cases, some diagnosed as scarlet fever, others as septic sore throat, 68 were traceable to a single raw milk supply, the others were contact cases. The same hemolytic streptococcus was isolated from them all. The milk was probably contaminated by the owners of the dairy, one of whom had had a middle-ear infection, the other a sore throat. For a detailed discussion of these streptococci, the reader may profitably consult Williams' book on the subject.[122]

Diphtheria. Diphtheria is an infection of the mucous membrane of the throat or nose, and is usually spread by droplet infection—that is, the bacteria may be carried to a distance of several feet in the drops of moisture exhaled in ordinary breathing, and considerably farther when impelled by a cough or sneeze. It may also be spread by articles contaminated directly or indirectly by mucous discharges. When milk is handled by a diphtheria patient, or a carrier, or a person in contact with a patient, the causative organism readily gains access to the milk in which it grows well without inducing any noticeable chemical change. Diphtheria infection can occur in wounds or raw surfaces as well as in the usual location in the mucous membrane. While cows are not susceptible to diphtheria in the ordinary sense, there are a few well established instances of milk contamination from a sore on the cow's udder. McSweeney and Morgan [62] in 1928 reported a small diphtheria epidemic traced to a dairy where the farmer's daughter was a carrier and *C. diphtheriae* was present as a secondary invader in cow-pox lesions on the cows.

Utensils washed in the farmhouse kitchen by a woman who also cared for a sick child have been known to carry enough bacteria to the milk to cause serious epidemics. A particularly interesting and instructive example is described in the Massachusetts State Board of Health Monthly Bulletin for May, 1907.[63]

Although notable outbreaks of diphtheria have been traced to milk, in general it is responsible for only a small proportion of cases compared to those due to contact or droplet infection or to contaminated toys, school supplies, and other objects. Crumbine reported only nine milk-borne epidemics in the United States and Canada in the years 1924–31, and stated that there was none in 1932.[18] There were, however, two in the United States in 1933.[19]

Poliomyelitis. It seems to be an established fact that the filterable virus which causes poliomyelitis is present in the secretions of the mouths and noses of patients, but its means of travel from one victim to the next remains an unsolved mystery. On purely epidemiological grounds, two outbreaks, one in Broadstairs, England, and one in Cortland, N. Y., have been traced to the milk supplies. It hardly seems likely, however, that this disease is generally so spread.

Typhoid fever. Milk-borne intestinal infections are a serious public health problem, and of these typhoid fever is the most common. About one-half of the milk-borne epidemics in the last few years have been typhoid. To most people it suggests the idea of polluted water, but like other excrementitious diseases it can be conveyed in a number of ways. As the primary seat of infection is the small intestine, the feces and frequently the urine are heavily infected throughout the duration of the fever and for a week or more after convalescence is established. Some individuals continue to harbor the organism for years and excrete it intermittently. Such persons, typhoid carriers, are a greater menace to the public health than patients incapacitated by the disease, particularly if they are food-handlers. Carriers are sometimes found who give no clinical history of typhoid fever, the original attack having been too light to be recognized. The infection can be transmitted by a patient or carrier to others by direct contact, but is usually spread by food or water contaminated directly or indirectly by excreta. Only habits of the most fastidious cleanliness would prevent a carrier from contaminating objects he handles. Excreta exposed on the ground or deposited in poorly constructed privies can be a source of pollution, since such material may be conveyed by flies, fowls, rats or other vermin to foodstuffs or utensils. If polluted water is used to wash or rinse cans the milk may be contaminated by the small residue left in the can.

Explosive outbreaks of typhoid are usually traceable to water or milk. When city water supplies were first established many such epidemics were traced to them; since that time, however, large scale purification plants have been installed in most cities, and where the water is irreproachable, milk will usually appear as the principal vehicle of epidemic typhoid. When large quantities of milk are mixed at central stations more noticeable epidemics might be expected, and yet large cities, getting their milk from a distance, are remarkably free from such outbreaks. The long haul and storage, during which a considerable number of the bacteria may die, and the dilution of the contaminated milk, are probably of some effect. Of far greater importance is the fact that pasteurization is more general in large city milk supplies than in small individual dairies. The 25 milk-borne typhoid epidemics in the United States in 1933 were all due to raw milk.[37] Typhoid fever in modern times occurs typically in small towns, villages and rural communities.

Paratyphoid. Paratyphoid organisms (*Salmonella*) are distinguished from the true typhoid bacterium (*Eberthella typhi*) by fermentation reactions and serological tests. They are disseminated by patients and carriers in much the same ways as the typhoid organism, though paratyphoid epidemics are more apt to be carried by milk or other foods than by water.

A fever very similar to typhoid is caused by certain members of this group, particularly by *Salmonella paratyphi* (*"B. paratyphosum A"*) and *Salmonella schottmuelleri* (*"B. paratyphosum B"*). This disease, more common abroad than in the United States, is sometimes spread by milk.

An outbreak of 150 cases at St. Catherine's, Ontario, in 1933, was spread by raw milk accidentally pumped into the cooler and bottler at the pasteurization plant.[61] Of far commoner occurrence in this country are the violent gastrointestinal disturbances, acute in onset, usually brief in duration and of low mortality, often alluded to incorrectly as "ptomaine poisoning." Such symptoms are generally the result of eating food containing other Salmonella organisms which are pathogenic for domestic animals or rodents as well as for humans. Milk products are as often responsible for such epidemics as liquid milk itself.

Food poisoning is not confined to the effects of paratyphoid organisms. Poisoning by persistent udder staphylococci in milk has been mentioned earlier in this chapter. There is on record one unquestionable case of *C. botulinum* in home-made cottage cheese, which caused the death of three persons. Linden, Turner and Thom [59] have reported instances of poisoning from well-aged hard cheeses heavily loaded with cocci sufficiently similar to *S. lactis* to have been overlooked as normal, were it not for the large number present. Other similar cases have since come to light. In many instances [70] when circumstantial evidence has pointed to milk or a milk product as the cause of an outbreak of poisoning, diligent search for bacteria of the paratyphoid type has been fruitless.

Infantile diarrhea. Infantile diarrhea or "summer complaint" presents a somewhat similar, though more complicated, problem and is of great importance as it is responsible for a considerable portion of the infant death rate, particularly in artificially fed babies. Investigators have never agreed upon a specific causal organism. In some cases bacteria of the colon, dysentery, paratyphoid, or *C. welchii* groups seem to be responsible. For example, of the two epidemics in the United States which have been traced to certified milk,[123] one was an outbreak of about 60 cases of gastroenteritis in infants and young children, due to contamination of the milk by a paratyphoid carrier.

There is considerable evidence that diarrhea may also be caused by indirect bacterial effect on the milk. The pioneer work of Flügge,[35] in 1894, pointed out that while infantile diarrhea increased in hot weather, the increase was many times as great in bottle-fed babies. As only a small proportion of the cases he studied could be explained by intestinal infection with known pathogens, he considered the possibility of poisoning by substances produced in the milk by saprophytes. He studied those organisms found in milk which survive heating and multiply at 25° to 30°. Milk in which certain aerobic spore-bearers had been allowed to multiply, showed little if any acidity or other obvious deterioration, but did produce diarrhea in young animals. He called attention to the irritating properties of various peptones and proteoses and urged that heated milk should be iced to inhibit multiplication of the organisms which survive heating and grow readily at ordinary summer temperatures.

Park and Holt,[76] in 1903, published the results of an extensive investigation on the incidence of this disease in tenement infants fed on milk of various degrees of freshness and purity, and prepared in various ways.

They concluded that heat and poor care were the primary factors, and that bacterial contamination of the general milk supply was secondary, unless excessive in amount or due to definitely pathogenic organisms. During cold weather the quality of the milk purchased made comparatively little difference, while in hot weather it made a great deal of difference whether the milk was fresh and of low count, and also whether or not it was heated before being fed to the baby. Even on milk with counts below 10,000 the results were noticeably better when the milk was heated. The best results were obtained with milk distributed at milk stations. The fact that this milk was properly modified and pasteurized in separate bottles for each feeding, and that the mothers who came for it received advice as to the care of infants, was of considerable effect, in the opinion of the authors, as well as the respect which tenement dwellers had for such milk and the pains they would take to keep it from contamination. Newsholme,[71] in England, stated that the chances of an infant's dying of diarrhea were quadrupled by artificial feeding. He came to the conclusion that "domestic contamination," that is, improper handling of milk in the home and its exposure to flies, was the principal cause of the trouble, rather than the presence of pathogenic bacteria in the milk when purchased. Scholberg and Wallis [98] report that in incubated raw milk, at rather early stages of bacterial multiplication, they found certain peptone-like substances capable of causing diarrhea by an effect on the pancreas. These substances were produced by types of organisms not ordinarily considered pathogenic for humans.

Many pediatricians have inclined to explain summer-complaint by the debilitating effect of the weather itself, coupled in the case of artificially fed infants with the difficulties caused by a diet imperfectly adapted to their powers of digestion. This explanation, while attractively simple, applies only to those cases where definite intestinal infection has been ruled out, and does not alter the fact that improvement of the milk supply has been often accompanied by enormous drops in infant mortality, notably in the New York tenement district after the establishment of the Nathan Straus milk depots in 1893. A particularly clear-cut case appeared in the children's institution on Randall's Island, New York City, where the infant mortality due to diarrhea and enteritis dropped from 44.36 to 19.80 after pasteurized milk was introduced, though it is said that no other improvement in regimen or sanitation was adopted.[120]

Preventability of milk-borne epidemics. An additional feature of milk-borne epidemics in general should be emphasized—their preventability. They do not occur when sufficient precautions are taken to make sure that the cows are healthy and that the milk is handled in a sanitary manner by persons free from infectious disease. This is evidenced by the fact that in this country only two epidemics spread by certified milk have been reported since 1892. To safeguard the enormous amounts of inexpensive milk required by large cities, where it is impossible to deliver the milk to the consumer until it is nearly if not quite a day old, pasteurization is necessary. This process, if properly carried out, is extremely

efficient, but has its limitations. It can not insure the consumer against undesirable products of excessive bacterial multiplication before the heat is applied, and of course does not protect the milk against subsequent contamination. Nevertheless, milk produced under reasonably clean conditions, adequately pasteurized while fresh, and kept sealed, will be free from pathogens.

Godfrey,[89] in an extensive survey of milk-borne epidemics in this country up to 1923, found record of but 16 that were alleged to have been caused by pasteurized milk. These he investigated in detail. In one case there was probably no connection between the milk supply and the epidemic; in four instances it was admitted that the milk was treated by the flash method or some other unapproved process; in one the thermographic records were "not available"; in five there was conclusive evidence of infection after pasteurization; and in one the evidence of subsequent contamination was very strong; thus only four outbreaks were left unexplained. In Crumbine's report for the period 1924-31,[18] which covers Canada as well as the United States, he lists only three cases as due to milk supposed to be pasteurized; in two the pasteurization was not properly done, in the third the information is inadequate. The same author in his report for the year 1932,[19] already cited, reports 30 epidemics, all due to raw milk except 2. In one of these the milk after pasteurization was bottled by a scarlet fever patient. There was also an outbreak of typhoid caused by ice cream made from a pasteurized mix, but the dealer may have been a typhoid carrier. The 39 epidemics listed by Frank for the United States in 1933 [37] were all due to raw milk. When we consider the large amounts of pasteurized milk sold, this indicates a frequency of infection which is negligible compared to that of ordinary raw milk. The problem of milk-borne disease is extremely important and somewhat complicated, but not insoluble.

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Chapter XI

The Metabolism and Growth of Bacteria in Milk

Introduction

Our knowledge of the metabolic processes of bacteria is limited for the most part to the food which organisms are able to use under certain conditions and the final products of the action of the organisms on that food. The intermediate processes and products are mostly a matter of theory. The metabolism of bacteria in very simple media is little understood and in a complex medium like milk still less is known about the metabolic changes.

In order to determine the action of a given organism on a medium all conditions of environment must be carefully controlled, for a variation in one or more of these conditions may change the action of the organism on the medium. Unfortunately it is very difficult to duplicate exactly in the laboratory the conditions which an organism may find in a given sample of normal raw milk. A study of the action of bacteria on milk is usually made in pure culture in sterilized milk or in a simpler culture medium; and the results of these experiments are used to determine the action of the organism on milk. There is no assurance, however, that these results will apply whenever that particular kind of organism grows in milk. Fresh and aseptically drawn samples of milk vary enough at times to cause variations in the action of bacteria.[100, 185] A pure culture of an organism may produce strikingly different amounts of acid in different milks[18, 229] and the organism may exhibit differences in microscopic appearance when grown in different milks.[213, 229] Variations in the suitability of milks as culture media may favor certain types of organisms and the milk is said to have a "disposition" toward a type of fermentation.[197] The most aseptically drawn milk usually contains more than one kind of bacterium. Organisms acting in association usually do not act as they do in pure culture, for they are apt to be either mutually helpful or antagonistic. Most decompositions in nature are the result of the action of a succession of organisms which grow partly on by-products of their predecessors. So while pure culture studies give an idea of the food requirements and normal action of an organism, they can not give a complete picture of what happens ordinarily in milk. Certain kinds of bacteria find conditions so favorable in milk, however, that at times they predominate and cause characteristic fermentations which are almost like their action in pure culture. The heated milk used in pure culture studies is undoubtedly different from unheated fresh milk. In laboratory studies

simpler culture media are used and the results are often applied to the very complex medium, milk. Different strains of the same species of organism vary among themselves in activity, resistance to adverse conditions and even in their ability to use certain foods. Variations in temperature, oxygen supply, surface tension, reaction and oxidation-reduction potential may produce varying results when a culture is acting on milk.

A discussion of the metabolism of bacteria in milk must, then, be very general in nature. Caution must be used in attempts to apply the results of the numerous experiments with milk organisms to the explanation of their metabolism in milk. All of these experiments help toward an understanding of the metabolism of bacteria in general and may help in the study of the action of organisms on milk.

Nutrition of Bacteria

General. Bacteria apparently must have their food in a comparatively simple form before they can synthesize it into cell compounds. The mineral elements may be taken in as simple salts. Proteins must be broken down to simpler nitrogen compounds before they are assimilated. Polysaccharides must be split to simple sugars and fats must first be hydrolyzed before these carbon compounds are available as energy sources. When a carbon compound is to be used as food for growth it probably must be in a very simple form. These simple assimilable substances which the organism uses in building up its cell structure are sometimes termed "building stones."

The exact form in which these various foods are assimilated by bacteria has not been definitely determined and probably varies with different organisms. Bacteria which are able to use ammonia as a sole source of nitrogen must be able to combine it with some simple carbon compound in order to start the synthesis of their cell proteins. It has been shown that *Bacterium pyocyaneum* [1] is apparently able to synthesize ammonia and pyruvic acid to alanine and then build more complex nitrogen compounds from the alanine. Most bacteria seem to be unable to use ammonia as their only source of nitrogen and require certain amino acids or even more complex nitrogen compounds. Ehrlich [108] believes, however, that substances simpler than the amino acids are used in the synthetic processes of the cell. Quastel [322] thinks that bacteria change carbon food substances to one or more definite compounds which serve as "building stones" for the manufacture of more complex carbon compounds. For *Bacillus coli* pyruvic acid may be such a simple carbon compound.

. It has been shown by a number of workers that bacteria, like higher organisms, are able to convert carbohydrates to fats, to use carbohydrates in building up nitrogen compounds, and to use nitrogen compounds as energy sources. It has also been noted that acetaldehyde is an intermediate product common to the decomposition of proteins, carbohydrates and fats. It has been supposed, therefore, that acetaldehyde, or its parent substance pyruvic acid, may occupy a central position in the change of one form of

food to another, and is important in all metabolic processes of the cell. This will be discussed in more detail under the decomposition of the various food substances of milk.

The food used by bacteria is sometimes classified as food for energy or food for growth. A single substance may be used for both purposes or may be used interchangeably for one or the other purpose depending upon conditions. In general with most organisms, carbohydrates or allied compounds usually are used as foods for energy, while the nitrogen and mineral compounds serve as foods for growth. Energy may result either from oxidation processes or from anaerobic decompositions. The carbon for growth may or may not come from the same source as the nitrogen. So little is known concerning the carbon compounds used in cell synthesis that the source of carbon for growth purposes is not discussed and the decomposition of carbon compounds is considered only as a source of energy. Comparatively large amounts of energy food are necessary, while food for growth purposes need be present only in small quantities. If a protein is being used only as a source of nitrogen, then small amounts of the protein are apt to be decomposed, but if it is being used as a source of energy large amounts will be broken down.

Kendall and Ishikawa [219] claim that "resting" bacteria initiate changes which tend to make available materials which they, as proliferating organisms, would subsequently use for their energy requirements.

The presence of a fermentable carbohydrate has been shown by Kendall [214] and his coworkers and by Walker and Winslow [465] to have a "sparing" effect on the proteins present. This seems to hold for many organisms but Raistrick and Clark,[329] Frazier and Rupp [119] and others have shown that some organisms break down more protein if they have a sufficient supply of available carbohydrate.

Milk as a culture medium. Milk would seem to be an ideal culture medium for bacteria. It is almost neutral in reaction and is well buffered. Its ash contains all of the mineral elements which are considered essential for the growth of bacteria. It contains a carbohydrate in the form of lactose and other carbon compounds, the fats and citrates. As sources of nitrogen it contains casein, lactalbumin and lactoglobulin, and very small amounts of simpler nitrogenous compounds.

Many bacteria do find milk a very favorable culture medium; but others can not grow well in it. A number of organisms can not split the disaccharide, lactose, and fewer can utilize citrates. Many organisms are unable to split casein, lactalbumin or lactoglobulin, even after the small amount of simple nitrogenous matter of milk has given the organisms a start. Organisms which can readily decompose lactose and utilize casein are likely to predominate at first; and the kind and amount of their byproducts will determine the action of their successors.

The decomposition of milk compounds may be divided into two types: the primary decompositions in which the original constituents of the milk are attacked, and secondary decompositions in which products of a previous decomposition are utilized.

When milk is to be used as a culture medium it is usually sterilized by heat, either by the intermittent method or by steam under pressure, and this heat treatment causes important changes in the milk. The amount of browning or caramelization produced varies with the heat treatment. Heating at autoclave temperatures, according to Orla-Jensen,[299] increases the hydrogen-ion concentration due to a combination of aldehyde groups of the carbohydrate with amino groups of the nitrogenous substances with the liberation of a corresponding number of carboxyl groups. Orla-Jensen points out that lactic rod-shaped bacteria which are favored by a pH of about 6, grow best in a milk which has received a strong heat treatment during sterilization so that the pH value has been dropped considerably, while lactic streptococci with an optimum pH of about 6.5 grow better in a less strongly heated milk. This worker also demonstrated that sugars are changed during the heat sterilization of culture media with the production of small amounts of methylglyoxal or other simple carbon compounds. Similar changes in the lactose probably occur during the autoclaving of milk. Milk which has been autoclaved may be so changed that proteolysis by bacteria is inhibited, according to Tarnanen [420] and Gorini.[187] Frazier and Rupp [120] found that autoclaving milk favors the action of the more active proteolytic organisms, but makes little difference in the action of the weakly proteolytic types.

Pasteurization seems to produce changes in milk which influence bacterial growth. It has been shown that increasing the temperature of pasteurization from 62.8° to 85° caused a decrease in the time required for coagulation by a lactic acid culture.[433] Bogdanoff [42] found that the growth of bacteria of the *S. lactis* and *B. casei* types was reduced in milk which had been pasteurized for 15 minutes at 45° to 55°, but was increased in milk which had been pasteurized at 60° or higher; the effect increased progressively with increases in temperature up to 120°, and acid production paralleled growth. Orla-Jensen and Jacobsen [305] found that bacteria grew best in milk which had been pasteurized previously at a high temperature; the effect was progressive as the pasteurization temperature was increased above 75°. The effect was assumed to be due to the destruction of the germicidal properties of the milk. Similar conclusions were reached by Hammer and Baker.[162]

The mineral requirements of bacteria. Bacteria use very small amounts of the minerals in their growth, traces sufficing in many instances. Because of this fact it is difficult to ascertain whether or not a given mineral is essential for bacterial growth. It has been supposed that the following elements are necessary for the growth of bacteria: [255] P, K, S, Fe, Ca and Mg. Experiments have indicated that S, K, Ca and Fe are not always essential but better growth is produced in their presence.[29] At any rate, all of the essential elements above named are found in milk in amounts sufficient for bacterial growth. Calcium and phosphorus which are tied up in the casein molecule are carried into solution when an acid fermentation takes place,[441] and the sulfur may be freed during the splitting of the casein molecule.

Little work has been done on the catalytic or coenzyme effect of minerals on the enzyme actions of bacteria; but it is known that such an effect is sometimes produced. Calcium is known to favor the action of bacterial as well as animal rennet.[119] The growth of *Bacterium coli* is stimulated by certain anions and cations,[188] and even salts which are ordinarily toxic may be stimulating in the proper concentration.[194] Magnesium and other metallic salts stimulate the lactic fermentation.[338] Phosphates are known to play an important part in the decomposition of sugars by some organisms. The addition of small quantities of iodine to milk increases the rate of growth of bacteria.[414]

Carbon metabolism in milk. The carbon compounds of milk include lactose, citrates and fats, together with the nitrogenous compounds casein, lactalbumin, lactoglobulin, and a few simpler nitrogenous substances. Since a carbohydrate, if fermentable, is more likely to be attacked than are other compounds, lactose is usually the chief source of carbon for the milk bacteria.

Lactose as a source of carbon. The disaccharide, lactose, can be hydrolyzed to one molecule of glucose and one of galactose. Some bacteria are said to possess an enzyme, lactase, which can split lactose in this way. Orla-Jensen [303] states that lactase is an endoenzyme of the "true lactics." Lactase has been reported in *Bacillus bulgaricus,*[85] *B. acidilactici,*[145] *Bacterium coli* and others.[461] It is sometimes claimed that certain organisms act directly on the lactose without first splitting it to simpler sugars; and most of the older discussions of the lactic acid fermentation follow this idea.

Of the two hexoses which result from the hydrolysis of lactose, the glucose is more readily fermented by most organisms than is the galactose. This fact may complicate the fermentation of the lactose in some cases.

Theories of fermentation. The first step in the decomposition of a hexose sugar, according to some workers,[226, 298] usually is the formation of a hexose phosphoric acid ester or zymophosphate, although it has been shown [291, 448, 457] that decomposition can take place without phosphorylation. The structure of the zymophosphate varies in different fermentations and with different hexoses. Varying proportions of hexose mono- and diphosphoric esters have been found [46, 345, 448] and among the esters which have been identified are: hexose-6-monophosphoric,[345] 1,6-diphosphoric [277] and 2,5-diphosphoric esters. Robinson and Morgan identified at least four different esters.[345] Bernhauer [33] states that only one of the three hydroxyl groups of orthophosphoric acid is esterified with an alcohol group of the sugar.

From the zymophosphate the hexose, e.g. glucose, is supposed to be liberated in a more reactive form, although experiments by Neuberg and Kobel on certain fermentations [290, 291] do not support this assumption. Harden,[171] however, states that in fermentation by dried yeast, added phosphate accelerates the reaction twenty fold, and that in the case of living yeast with a high rate of fermentation the balance of enzymes in the cells is such that the supply of phosphate is maintained at an optimum and

added phosphate causes no acceleration in the rate of the reaction. Virtanen and Karström [453] found that the lactic acid fermentation of glucose occurred in two phases. In the first, phosphorus binding was rapid and lactic acid production was slow; in the second phase, after half of the glucose had been decomposed, a liberation of phosphorus and a rapid production of lactic acid took place. Virtanen [457] has reported a type of fermentation in which part of the hexose is not phosphorylated but is decomposed into a two-carbon chain and a four-carbon chain, yielding acetaldehyde and succinic acid. This reaction was induced by dried cells in the presence of toluol and absence of coenzyme.

When the zymophosphate is broken down with liberation of the phosphate, the hexose part of the molecule is converted to one or more intermediate products the existence of which is theoretical. Thus a three-carbon compound, dihydroxyacetone, glyceric aldehyde or methyl glyoxal, is supposedly formed from glucose. A rearrangement of the methyl glyoxal molecule may give lactic acid. In the case of the alcoholic and most other fermentations, however, pyruvic acid is the next intermediate product and this is decarboxylated to acetaldehyde. Methyl glyoxal, pyruvic acid and acetaldehyde have been actually identified as intermediate products by various workers. There is considerable disagreement, however, about the succeeding steps. The two principal modern theories of fermentation are that of Kluyver and Donker and that of Neuberg and his coworkers.

Kluyver and Donker [222] first based their theory on the protoplasmic dehydrogenation-hydrogenation theory of Wieland.[474] In their early work they [222, 228] considered the cell protoplasm as a hydrogen transporting catalyst and believed that the course of the reaction was determined by the affinities of the cell protoplasm for hydrogen and oxygen. After protoplasm had taken up hydrogen it was "regenerated" through the loss of the hydrogen to hydrogen acceptors such as the substrate, molecular oxygen, intermediate compounds, or even foreign substances introduced into the fermenting fluid. Several workers have shown, however, that dead cells can take part in certain decomposition processes.

In a later formulation of the above theory Kluyver and his coworkers [224, 225] have stressed the importance of hydrogen acceptors and have made little or no mention of the action of cell protoplasm. The fermentation is supposed to take place by means of a series of coupled dehydrogenation-hydrogenation reactions in which the fermenting compound. or a chemical group contained in it, becomes catalyzed or activated in such a way that a hydrogen atom becomes labile (**H** in reactions below indicates labile hydrogen), and is removed to an acceptor which may be another group in the same molecule or part of a different molecule.

The steps in the fermentation of a sugar like glucose, according to the theories of Kluyver and his coworkers, are as follows: [226]

1. Formation of glyceric aldehyde by phosphorylation and subsequent oxidation-reduction:

(a) $C_6H_{12}O_6 + PO_4R_2H \rightarrow C_6H_{11}O_5(PO_4R_2) + H_2O$
(b) $C_6H_{11}O_5(PO_4R_2) \rightarrow C_3H_6O_3$ (glyceric aldehyde) $+ C_3H_5O_2(PO_4R_2)$
(c) $C_3H_5O_2(PO_4R_2) + H_2O \rightarrow C_3H_6O_3 + PO_4R_2H$

2. Intermolecular hydrogenation of glyceric aldehyde to glycerol and intramolecular oxidation-reduction of glyceric aldehyde to methyl glyoxal hydrate:

(a) $CH_2OH.CHOH.CHO$ (glyceric aldehyde) $+ 2H \rightarrow$
$CH_2OH.CHOH.CH_2OH$ (glycerol)
(b) $CH_2OH.CHOH.CHO$ (glyceric aldehyde) $\rightarrow$
$CH_3.CO.CH(OH)(OH)$ (methyl glyoxal hydrate)

3. Change of methyl glyoxal hydrate to its stable isomer, lactic acid, to acetaldehyde and formic acid, or to pyruvic acid and acetaldehyde:

(a) $CH_3.CO.CH(OH)(OH) \rightarrow CH_3.CHOH.COOH$ (lactic acid)
(b) $CH_3.CO.CH(OH)(OH) \rightarrow$
$CH_3.CHO$ (acetaldehyde) $+ H.COOH$ (formic acid)
(c) $CH_3.CO.CH(OH)(OH) +$ acceptor $\rightarrow$
acceptor $<^{H}_{H} + CH_3.CO.COOH$ (pyruvic acid)
$CH_3.CO.COOH \rightarrow CH_3.CHO$ (acetaldehyde) $+ CO_2$

4. Dehydrogenation reactions:

(a) $H.COOH \rightarrow HCOOH \rightarrow CO_2 + 2H$
(b) $CH_3.CHO + H_2O \rightleftarrows CH_3.CH(OH)(OH) \rightarrow$
$CH_3.COOH$ (acetic acid) $+ 2H$

5. Hydrogenation reactions:

(a) $2H \rightarrow H_2$ (gas)
(b) $2H + O$ (activated) $\rightarrow H_2O$
(c) $CH_2OH.CHOH.CHO$ (glyceric aldehyde) $+ 2H \rightarrow$
$CH_2OH.CHOH.CH_2OH$ (glycerol)
(d) $CH_3.CHOH.COOH$ (lactic acid) $+ 2H \rightarrow$
$CH_3.CH_2.COOH$ (propionic acid)
(e) $CH_3.CHO + 2H \rightarrow CH_3.CH_2OH$ (ethyl alcohol)
(f) $CH_3.CO.CHOH.CH_3$ (acetylmethylcarbinol) $+ 2H \rightarrow$
$CH_3.CHOH.CHOH.CH_3$ (2,3-butylene glycol)

6. Condensation reactions; the formation of acetylmethylcarbinol, butyric acid and acetone is accounted for by this type of reaction:

(a) $2CH_3.CHO \rightarrow CH_3.CO.CHOH.CH_3$ (acetylmethylcarbinol)
(b) $2CH_3.CHO \rightarrow CH_3.CHOH.CH_2.CHO$ (acetaldol) $\rightleftarrows$
$CH_3.CH{=}CH.CH(OH)_2 \rightarrow CH_3.CH_2.CH_2.COOH$ (butyric acid)
(c) $2CH_3.COOH \rightarrow H_2O + CH_3.CO.CH_2.COOH$ (acetyl acetic acid) $\rightarrow$
$CO_2 + CH_3.CO.CH_3$ (acetone)

Differences in the end products within a group of bacteria are not essential ones since the whole course of fermentation may be changed by the suppression of one of the earlier stages. The course of the reaction, or the type of fermentation, varies for different organisms; however, the internal mechanism of the reactions, through the formation of methyl glyoxal or its hydrate, is the same in most other fermentations as in the alcoholic fermentation. The course of the reaction for certain cells is supposed to be determined by the power of enzymes or catalytic agents of the cells to activate hydrogen atoms in the materials in the substrate. The

differences between the "true lactics" and other lactics are thus said to be due to the non-appearance of reactions with the true lactics which do occur with the colon-aerogenes organisms, for example.

It will be noted that the theories of Kluyver and his associates can be connected with the observations of Clark and his colleagues [56] on the oxidation-reduction potentials of bacteria. The latter suggest "that there must be some correlation between the characteristic potentials and the metabolism characteristic of species." While they do not entirely agree with the theories of Kluyver as presented, they believe that the fundamental idea can be expressed in terms of experimental data.

The fermentation theories of Neuberg and his coworkers [293, 295] are based chiefly on Cannizzaro rearrangements, whereas Kluyver considers the mechanism of the conversion as transferences of activated hydrogen or coupled dehydrogenation-hydrogenation reactions. According to Neuberg, glucose is changed to one or more hypothetical three-carbon compounds after the formation of the zymophosphate. The hexose-phosphoric acid ester is split by a phosphatase enzyme. Methyl glyoxal is considered the first definite three-carbon product and may be formed by the splitting out of two molecules of water from the hexose molecule. Methyl glyoxal aldol may be an intermediate compound. The existence of dioxyacetone and glyceric aldehyde as intermediate products is doubted. The methyl glyoxal may be stabilized as lactic acid [9, 288, 293] or may go through repeated Cannizzaro rearrangements to form various fermentation products. The next intermediate product is pyruvic acid which may be reduced to form glycerol or decarboxylated to form acetaldehyde and carbon dioxide. Pyruvic acid may be almost quantitatively changed under certain conditions to acetic acid, carbon dioxide and hydrogen [289] or to acetic and formic acids.[72] It has been noted above that acetaldehyde is apparently an important intermediate product in most processes of dissimilation. A Cannizzaro reaction is supposed to take place between the two aldehydes, methyl glyoxal and acetaldehyde, with the formation of ethyl alcohol and more pyruvic acid. From the latter compound by decarboxylation more acetaldehyde and carbon dioxide are formed; and there is thus a continuous formation and decomposition of pyruvic acid which, like the methyl glyoxal, for this reason can not accumulate. At the end of the process a little acetaldehyde would remain and traces are always detectable.

Neuberg and his coworkers [293] have postulated five different types of fermentations as indicated by the following equations:

First type, the usual alcoholic fermentation under anaerobic conditions:

1. $C_6H_{12}O_6 - 2H_2O \rightarrow CH_3.CO.COH$ (methyl glyoxal)

2. $$\begin{array}{ccccc} CH_2{=}C(OH).COH & + H_2O + & H_2 & \rightarrow & CH_2OH.CHOH.CH_2.OH \text{ (glycerol)} \\ + & & \| & & + \\ CH_2{=}C(OH).COH & & O & & CH_2{=}COH.COOH \text{ (pyruvic acid)} \end{array}$$

3. $CH_3.CO.COOH \rightarrow CO_2 + CH_3.COH$ (acetaldehyde)

4. $$\begin{array}{ccccc} CH_3.CO.COH & + & O & \rightarrow & CH_3.CO.COOH \text{ (pyruvic acid)} \\ + & & \| & & + \\ CH_3.COH & & H_2 & & CH_3.CH_2OH \text{ (ethyl alcohol)} \end{array}$$

Second type, if the acetaldehyde is removed or fixed:

$$C_6H_{12}O_6 \rightarrow CH_3.CHO + CO_2 + CH_2OH.CHOH.CH_2OH \text{ (glycerol)}$$

Third type, in weakly alkaline medium:

$$2C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH \text{ (ethyl alcohol)} + CH_3.COOH \text{ (acetic acid)} + 2CO_2 + 2CH_2OH.CHOH.CH_2OH \text{ (glycerol)}$$

Fourth type, pyruvic acid and glycerol from sugar:

$$C_6H_{12}O_6 \rightarrow CH_3.CO.COOH + CH_2OH.CHOH.CH_2OH$$

Fifth type, simple desmolytic decomposition to methyl glyoxal:

$$C_6H_{12}O_6 \rightarrow 2H_2O + 2CH_3.CO.COH$$

That a Cannizzaro reaction between two aldehydes is possible in fermentation has been shown by Neuberg and Reinfurth [287] and others.[269, 293, 297] When two molecules of acetaldehyde combine by this "carbolization" process, acetylmethylcarbinol is formed. This is apt to take place in an excess of acetaldehyde; but usually there is enough labile hydrogen present to reduce the acetaldehyde to alcohol. Acetic acid may also be formed from acetaldehyde; [283] indeed the latter has been found to be an intermediate product in the oxidation of alcohol to acetic acid by bacteria.[284] Acetaldehyde and pyruvic acid are also intermediate products between sugar and butyric acid or butyl alcohol.[285]

The theories of Neuberg et al. involve a series of alternate oxidations and reductions, and the oxidation-reduction potential influences the course of the fermentation.[287] Since the hydrogen used in the reductions is not in the free state but requires an acceptor, some of the intermediate products are assumed to be acceptors, such as the acetaldehyde and the glyceric aldehyde in the equations above.

Kostytschew [288] disagrees with the theories of Neuberg, denies the existence of the enzymes reductase, mutase, carboxylase, carboligase, and claims that a mixed Cannizzaro reaction is improbable. He thinks that the whole fermentation process is the result of a very few enzymes. A specially named enzyme has been suggested for nearly every chemical change produced in fermentation processes. Space will not permit a discussion of these enzymes.

The two fermentation theories which have been roughly outlined above are still being expanded. Experimental data are being advanced in proof of each theory, but are insufficient, as yet, to warrant definite conclusions. Various modifications of the above theories have been suggested by Gottschalk,[188] Schoen,[371] Levene [252] and others.

According to some workers the fermentability of a substance is dependent on whether the bacteria can "activate" the substance and whether they can break it down to a compound which can be used in synthesizing cell compounds. The stereochemical form of a compound has been shown to determine its fermentability by an organism.[252, 324, 325, 371]

The lactic acid fermentation. The present theories for the forma-

tion of lactic acid from sugar have been briefly discussed above. It was noted that, according to most workers, bacteria split lactose by means of an enzyme, lactase, into glucose and galactose. Bacterial lactase is apparently an endoenzyme and consequently only that amount of the lactose which is fermented to acid is hydrolyzed while the remaining lactose is untouched.[303] The first stages in the lactic fermentation, according to Virtanen,[449] Neuberg and his coworkers [293] and others, are in general the same as the first stages in the propionic and alcoholic fermentations and lead to the formation of a three-carbon compound. In the presence of phosphates, as in milk, the hexose sugar may be changed to a hexose-diphosphate or zymophosphate as the first stage in decomposition. Virtanen [447] has shown that *B. casei* ε and *S. lactis* form a zymophosphate as the first step in the lactic fermentation. The fermentation is slow while phosphorylation is taking place, but later when the phosphate is being set free, the production of lactic acid is much more rapid.[453] The glucose (or other hexose) is supposedly released from the phosphoric ester in a very reactive form and, according to theory, goes to two molecules of a three-carbon compound, usually supposed to be methyl glyoxal (or glyceric aldehyde). The labile methyl glyoxal is stabilized as lactic acid:

$$CH_3.CO.CHO + H_2O = CH_3.CHOH.COOH \text{ (lactic acid)}$$

Buchanan and Fulmer [52] (Vol. III, p. 133) consider this change as a combination of methyl glyoxal with a molecule of water, followed by a dehydrogenation of the aldehyde group, with the ketone group of the same molecule acting as a hydrogen acceptor.

While Neuberg and Gorr [288] have reported that a *Lactobacillus* and *Bacterium coli* are able to convert methyl glyoxal to lactic acid, Virtanen and Karström [450, 451] claim that neither *S. lactis* nor *B. casei* ε can convert methyl glyoxal, dioxyacetone, glyceric aldehyde or pyruvic acid to lactic acid. Some workers believe that phosphoglyceric acid and pyruvic acid, and not methyl glyoxal, are intermediate products in the lactic fermentation.

Under favorable conditions the so-called "true lactics," like *Streptococcus lactis* and *Lactobacillus bulgaricus,* ferment the sugar chiefly to lactic acid with small amounts of other by-products. These by-products are considered by some to result from the respiratory processes of the cells. The chief source of energy for these organisms is, however, supposed to result from the formation of the lactic acid either in the presence or absence of free oxygen.[287] According to Suzuki, Hastings and Hart [415] 90 to 98 per cent of the lactose fermented goes to lactic acid and the remainder to alcohols, aldehydes and esters. Orla-Jensen [303] states that the true lactics form a little succinic acid, some acetic acid, traces of propionic acid and a little carbon dioxide. Bertrand and Weisweiler [34] report also traces of formic acid with *L. bulgaricus.* The kind and relative amount of these by-products vary with conditions. Under unfavorable conditions the more acetic acid is formed [20] and under favorable conditions the more carbon dioxide. There has been a controversy between Richet [339]

and Lumière [257] as to whether the lactic fermentation is irregular in the presence of antiseptics.

The following organisms have been reported to produce predominantly *d*-lactic acid: *S. lactis*,[178, 196, 5] *S. pyogenes* and closely related streptococci,[196] *S. liquefaciens*, some of the acid-proteolytic cocci,[195] *S. cremoris* [238] and *L. thermophilus*.[57] Lactic acid mostly in the *l*-form is said to be produced by: the aroma-forming streptococci or beta-cocci,[96, 308] *S. kefir*,[196] *S. thermophilus*, *Tbm. lactis*, *Tbm. bulgaricum*,[305] and *L. leichmanni*.[5] Mixtures of *d*- and *l*-lactic acid are said to result from the action of some acid-proteolytic cocci,[195] *L. bulgaricus*,[34, 84] *Tbm. yoghurt*, *Tbm. helveticum*,[305] *Sbm. casei*,[387] *C. welchii*, *C. butyricum* [203] and *L. acidophilus*.[84, 204] In the mixture of *d*- and *l*-lactic acid, the *l*-form was found to predominate after growth of cultures of *L. bulgaricus*,[34] and the *d*-form with *Sbm. casei*,[387] *C. welchii* [203] and *L. acidophilus*.[204] Some workers believe that the form of lactic acid produced is constant for an organism; other workers state that form may vary with changes in conditions of growth. An organism growing with another organism may produce a different form of lactic acid from what it would produce growing alone, even if the second organism does not produce lactic acid.[314]

It is supposed that the lactic acid formed by the true lactics is not further attacked by them; but Orla-Jensen [303] states that if there is no more sugar present than the lactic organisms can easily ferment, part of the lactic acid formed will be further decomposed. He says [302] that *Bacillus casei* α can act on lactic and succinic acids and further decompose them, while the ϵ type is unable to decompose lactic acid.

The acid fermentation of lactose by the "pseudo-lactics" or colon-aerogenes group has been studied to a greater extent than that by the true lactics. Sugar fermentation by *Escherichia coli* in particular has been the subject of the most experimental work. The final products of the action of *E. coli* on glucose are chiefly lactic acid, acetic acid, formic acid, alcohol, succinic acid, carbon dioxide and hydrogen.[169] Traces of other substances may be present due to secondary decompositions.

Although these organisms are called lactics, the amount of lactic acid formed is usually considerably less than one-third of the total products from a sugar fermentation. Most modern theorists agree that the formation of lactic acid is separate from the formation of the other by-products.[8, 143] According to the theories discussed above, the lactic acid would have an origin similar to that of the lactic acid produced by the true lactics, while the other by-products would result from a different series of changes. Virtanen [448] states that the sugar is not esterified in the colon-aerogenes fermentation and therefore no coenzyme is necessary. According to some writers [288] sugar fermentation by *E. coli* is similar to that by yeasts and the theories which apply to the alcoholic fermentation can be applied to the *E. coli* fermentation. The formation of acetic and formic acids, alcohol, carbon dioxide and hydrogen can be accounted for by either of the general fermentation theories discussed above. Harden [170] considered lactic and acetic acids, alcohol, hydrogen and carbon dioxide

as primary products of fermentation and the other products as secondary. He expressed the fermentation of glucose by *Bacillus coli communis* as follows:

$$2C_6H_{12}O_6 + H_2O \rightarrow 2CH_3.CHOH.COOH + CH_3.CH_2.OH + 2CO_2 + 2H_2$$

Neuberg and Gorr [288] say that this equation no longer holds, but that according to present knowledge the reactions should be expressed thus:

$$2C_6H_{12}O_6 \rightarrow \underset{\text{(lactic)}}{2CH_3.CHOH.COOH} + 2CH_3.CO.COOH + 2H_2$$

$$2CH_3.CO.COOH \rightarrow \underset{\text{(acetaldehyde)}}{2CH_3.CHO} + 2CO_2$$

$$2CH_3.CHO + H_2O \rightarrow \underset{\text{(ethyl alcohol)}}{CH_3.CH_2.OH} + \underset{\text{(acetic acid)}}{CH_3.COOH}$$

Methyl glyoxal is supposed to be an intermediate product between the glucose and the lactic or pyruvic acid. Aubel [9] discusses the energy changes concerned in these processes and concludes that most of the energy obtained by *Bacterium coli* is obtained from the decomposition to lactic acid and that part of this energy is used in the other part of the sugar decomposition (through pyruvic acid) which is a necessary step in the synthetic processes of the cell.

In terms of the Kluyver and Donker theory the final reactions in the *E. coli* fermentation would be the result of reductions and oxidations due to the transportation and activation of hydrogen by the cell. Thus if acetaldehyde were to serve as acceptor for hydrogen, ethyl alcohol would result. If acetaldehyde were oxidized by the removal of hydrogen from water by the protoplasm, acetic acid would result.

Organisms of the colon-aerogenes group are able to decompose further some of their primary fermentation products. Lactic, succinic [236] and formic [308] acids can be broken down by both *E. coli* and *A. aerogenes*. Rona and Nicolai[355] report that under anaerobic conditions glucose is split by *Bacterium coli* to two molecules of fixed (lactic) acid, and that this acid is used in respiratory processes when aerobic conditions are introduced.

The same theories of fermentation may be applied to the action of other groups of bacteria on lactose in explanation of the formation of lactic and acetic acids and other by-products. Orla-Jensen classes all the sugar-fermenting micrococci and sarcinae of milk as "tetracocci" and states that they form some lactic acid and more acetic acid.[303] At times the lactic acid is in very small amounts and part of the sugar is probably broken down to carbon dioxide and water. Many of these organisms are also able to ferment organic acids. Certain of the gram-positive rod-shaped bacteria which are not classed as true lactics act on sugar much as do the tetracocci.[303] In a study of the cheese streptococci other than *S. lactis,* Evans [113] found a *"Streptococcus X"* which produces large amounts of acetic acid. *S. kefir* produces lactic acid, acetic acid, carbon dioxide and alcohol.

The propionic acid fermentation. A great variety of organisms form propionic acid as a minor by-product of fermentation, but only a limited number produce it as one of the chief end-products. The true lactics produce traces of propionic acid in their fermentation of sugar [303] and the aroma-formers of Hammer [159] form some propionic acid. Propionic acid is said to result sometimes from protein decomposition and may be due to the deaminization of the amino acid, alanine.[184] Some aerobic rods produce propionic acid during a fermentation but it is supposed to result from protein decomposition. Some of the proteolytic organisms of milk, like the potato bacilli,[469] form propionic acid and at the same time butyric acid from the casein.

The organisms which are generally called the propionic acid bacteria are rod-shaped bacteria which grow anaerobically or in reduced oxygen tension. The four chief representatives of this group have been named *Bacterium acidi propionici a, b, c,* and *d,* respectively. Propionic fermentation by any of these organisms is not usually of importance in milk, but may be important in Swiss or other cheese.

The propionic acid organisms ferment either lactose or lactates with the formation of propionic and acetic acids and carbon dioxide.[381, 469] Virtanen [444] found that a propionic bacterium isolated from Swiss cheese gave two molecules of propionic acid to one of acetic acid in a well aerated calcium lactate medium:

$$\underset{\text{(lactic acid)}}{3CH_3.CHOH.COOH} \rightarrow \underset{\text{(propionic)}}{2CH_3.CH_2.COOH} + \underset{\text{(acetic)}}{CH_3.COOH} + CO_2 + H_2O$$

But under anaerobic conditions the ratio was three molecules of propionic acid to one of acetic.

Kluyver [226] states that the fermentation of lactic acid to propionic acid includes these reactions: (a) a dehydrogenation of lactic acid to pyruvic acid; (b) a reaction of the latter with water to form acetic acid, carbon dioxide and labile hydrogen; and (c) a hydrogenation of lactic acid directly to propionic acid (H indicates labile hydrogen):

$$\text{(a)}\quad CH_3.CHOH.COOH \text{ (lactic acid)} \rightarrow CH_3.CO.COOH \text{ (pyruvic acid)} + 2H$$

$$\text{(b)}\quad CH_3.CO.COOH \text{ (pyruvic acid)} + H_2O \rightleftarrows CH_3.C(OH)(OH).COOH \rightarrow CH_3COOH \text{ (acetic acid)} + CO_2 + 2H$$

$$\text{(c)}\quad CH_3.CHOH.COOH \text{ (lactic acid)} + 2H \rightarrow CH_3.CH_2.COOH \text{ (propionic acid)} + H_2O$$

Virtanen [457] believes that in the decomposition of glucose by propionic acid bacteria, as well as by *B. coli,* the glucose is split in two ways. Part of the glucose goes to a two-carbon compound, acetaldehyde, and a four-carbon compound which yields succinic acid. The acetaldehyde is changed to acetic acid. In the presence of living cells the balance of the glucose goes through the monophosphate, methyl glyoxal and lactic acid stages, and the latter compound goes to propionic and acetic acids and carbon dioxide.

Foote, Fred and Peterson [118] believe that a molecule of hexose is changed to succinic acid, acetaldehyde and carbon dioxide, and that an

oxidation of acetaldehyde to acetic acid is accompanied by a reduction of lactic acid to propionic acid.

The butyric acid fermentation. There are three possible sources of butyric acid,—the proteins, fats and carbohydrates. Butyric acid may result from the decomposition of various amino acids [184] or from the hydrolysis of fats. The so-called "true" butyric acid fermentation, however, is that of carbohydrates or their derivatives by anaerobic, rod-shaped bacteria. These fermentative bacteria are chiefly of two kinds, a motile type, like *Clostridium butyricum* (*Bacillus amylobacter*) and a non-motile type like *Clostridium welchii.*

Clostridium putrificum which is not considered a "true butyric" organism is a putrefactive anaerobic rod. It produces butyric acid chiefly by protein decomposition. Some of the aerobic proteolytic organisms also produce butyric acid as one of the final products of protein decomposition.

The true butyric acid bacteria ferment sugar with the production of butyric, lactic, formic and acetic acids [469] and carbon dioxide and hydrogen. Small amounts of valeric acid or alcohol may be formed. The non-motile type of organism curdles milk by the formation of the lactic and butyric acids. The motile type forms more butyric acid and less lactic.

The butyric acid fermentation may follow or be coincident with the lactic fermentation, since lactates are readily fermented to butyric acid. Some of the butyric bacteria seem to be unable to break down lactose and must act in association with some lactose-fermenting organism, like *S. lactis.*

The theories of fermentation agree that butyric acid must be a condensation product of one of the intermediate compounds. According to Kluyver and Donker the butyric acid may be formed by condensation of the intermediate compound, acetaldehyde:

$$2CH_3.CHO \rightarrow CH_3.CHOH.CH_2.CHO \rightarrow CH_3.CH_2.CH_2.COOH \text{ (butyric acid)}$$

A preliminary product in the fermentation [226] may be methyl glyoxal hydrate which goes to acetaldehyde and formic acid; some of the acetaldehyde may combine with water to form acetic acid and hydrogen and the formic acid may go to carbon dioxide and hydrogen. Considerable hydrogen is present in the gases formed in the butyric fermentation in cheese and other dairy products.[142, 148] Neuberg and Arinstein [286] point out that pyruvic acid is not only the mother substance of acetaldehyde but also of butyric acid. They give the following equations:

$$(1)\ C_6H_{12}O_6 \text{ (sugar)} \rightarrow 2CH_3.CO.COOH \text{ (pyruvic acid)} + 4H$$

$$(2)\ 2CH_3.CO.COOH \rightarrow \begin{array}{l} CH_3.C(OH).COOH \\ \quad\quad | \\ \quad\quad CH_2.CO.COOH \end{array} \text{ (or } C_6H_8O_6)$$

$$(3)\ C_6H_8O_6 \rightarrow 2CO_2 + CH_3.CH_2.CH_2.COOH \text{ (butyric acid)}$$

If lactic acid is fermented it goes to pyruvic acid and through the same succeeding stages.

The alcoholic fermentation. In the alcoholic fermentation yeasts ferment sugars almost quantitatively to form carbon dioxide and ethyl

alcohol in equimolecular proportions. The present theories of alcoholic fermentation have been discussed above under the general theories (p. 311). According to the Kluyver and Donker theory as described by Kluyver,[226] alcohol results from the reduction of acetaldehyde, and this compound is derived, along with carbon dioxide, from pyruvic acid; the latter is supposed to be formed by a dehydrogenation of methyl glyoxal hydrate; the hydrogen thus released is used in converting the acetaldehyde to ethyl alcohol. The reaction is of the hydrogen transference type. Phosphorylation precedes the formation of methyl glyoxal hydrate, as in most sugar fermentations.

Neuberg and Kobel [292] state that methyl glyoxal, pyruvic acid, and acetaldehyde occur as intermediate products and can be isolated by Neuberg's fixation method in 80 to 100 per cent yield. According to Neuberg's theory an oxidation-reduction occurs between methyl glyoxal and acetaldehyde, yielding pyruvic acid and ethyl alcohol; the pyruvic acid is decarboxylated to acetaldehyde which reacts with more of the desmolytically-formed methyl glyoxal to form more pyruvic acid and ethyl alcohol. According to Lebedew [248] the splitting of the hexose results in an active form of glyceric aldehyde as the first three-carbon compound and this is changed to methyl glyoxal.

A number of bacteria produce alcohol as one of their end-products but it is usually a minor product. The organisms of the colon-aerogenes group produce varying amounts of alcohol; and it has been noted above that the fermentation of sugar by this group is supposed to be very much like the alcoholic fermentation by yeasts.

Most ordinary yeasts are unable to produce an alcoholic fermentation in milk, due to the fact that they are unable to split lactose. A few of the Saccharomyces, however, and many of the Torulae are lactose-fermenting and are important in the production of some of the fermented milks. The fermentation is similar to other alcoholic fermentations in that alcohol and carbon dioxide are the chief end products, together with small quantities of volatile and non-volatile acids.

Citric acid as a source of carbon. The small amount of citric acid in milk, which is present chiefly as citrates of sodium and potassium,[440] may serve as a source of carbon for some bacteria. That these citrates in milk are attacked was shown by Bosworth and Prucha [45] who observed that the citric acid of milk disappears during the normal souring process. Kickinger [220] has shown that the citrates also decrease in amount in milk which has been boiled or pasteurized or sterilized by the intermittent method. It was demonstrated that this decrease in citric acid is not due to the heat but to the action of bacteria which survive the heating process. In the case of intermittent sterilization by heat there is a decrease after the first and second heating but none after the third.

There is some disagreement as to which organisms attack the citric acid in milk. Bosworth and Prucha [45] attribute the destruction of citric acid in souring milk to the action of *Bact. lactis aerogenes,* which was the only citrate-fermenting organism they found among the common milk

organisms. Kickinger [220] in his work on heated milk found that bacteria like *Bact. subtilis, Bact. mesentericus vulgatus* and *Proteus vulgaris* were responsible for the decrease in the citric acid. Hastings, Mansfield and Helz [177] point out that organisms which ferment citric acid in milk do not necessarily ferment it in a different organic medium, and that organisms which utilize citric acid in an inorganic medium with no other source of carbon do not necessarily ferment citric acid in milk. This is another example of the fact that the action of organisms in milk may be very different from their action in other culture media. According to these workers the nature of the available carbon is probably the determining factor in citric acid fermentation. They found that the citric acid in milk is fermented by *Escherichia coli, Aerobacter cloacae, S. citrovorus* and *S. paracitrovorus* and is not attacked by *S. lactis, L. casei, L. acidophilus, Aerobacter aerogenes,* casein digesters and lactose-fermenting yeasts.

Lampitt and Bogod [242] found that thermophilic bacteria cause little or no destruction of citric acid in milk, and that *L. bulgaricus* growing in milk at 42° decomposes citric acid and increases the amount of free fatty acids, but does not do so at 18° or 50°. These changes are slower in pasteurized than in raw milk. Mussill [281] found that the decrease in citric acid in stored milk is due entirely to bacteria, that *B. coli* is a most important factor in citric acid decomposition, and that certain spore-formers can also utilize it. Lampitt and Bogod [248] found that *E. coli* destroys citric acid more rapidly than does *S. lactis* or *A. aerogenes.*

Orla-Jensen and his coworkers [304] report that the organisms which they classify as betacocci can ferment citric acid. Hammer's aroma-formers, *Streptococcus citrovorus* and *S. paracitrovorus,* which are found in lactic starters, would fall in the same group, and Hammer and his associates [158, 271] report that these organisms ferment the citric acid of milk with the production of volatile acids, carbon dioxide and small amounts of the aroma constituents, acetylmethylcarbinol and biacetyl. Increased acid, either added or formed by *S. lactis,* stimulates the decomposition of citric acid and changes the proportion of various end-products. At a low acidity citric acid is changed slowly and the proportion of volatile acids, chiefly acetic, is high. At a higher acidity more of the carbinol is formed and the rate of decomposition of citric acid is increased regardless of the kind of acid added.

Another group of milk bacteria which can ferment citrates is the alkali-forming group. It was found by Ayres, Rupp and Johnson [12] that these organisms, which produce an alkaline reaction in milk without visible signs of peptonization, are able to ferment citrates with the production of carbonates and small amounts of other organic acids.

Although the end-products from citrate fermentation by different kinds of organisms may vary somewhat, the main reaction usually seems to be the oxidation of citric acid or citrates with the production of carbon dioxide and acetic acid. Bosworth and Prucha [44] found that *Bact. lactis aerogenes* decomposed one molecule of citric acid into two molecules of acetic acid and of carbon dioxide:

$$2C_6H_8O_7 \text{ (citric acid)} \rightarrow 4CO_2 + 4CH_3.COOH \text{ (acetic acid)}$$

The aroma-forming organisms of Hammer[158] produced volatile acids which were chiefly acetic and carbonic. The alkali-forming bacteria of milk[9] ferment citrates to organic acids in addition to the carbonates. Some of the Salmonella group of bacteria form succinic acid from citrates in addition to acetic acid and carbon dioxide.[48]

Fat as a source of carbon. The butterfat of milk is another possible source of carbon for the bacteria. But the fats do not seem to be as readily attacked by the bacteria as are the sugar and proteins. This may be due in part to the fact that the fats are not in solution in the milk serum but are in a state of emulsion with each fat globule surrounded by more or less of a layer of adsorbed matter. Then, too, bacterial lipases are usually active only in neutral or alkaline reactions and are inactive in the acid reaction which usually obtains when milk is acted on by bacteria.

According to some writers[335] milk as drawn contains a small amount of lipase which under favorable conditions slowly acts on the milk fat. It has not been shown whether or not this slight action may aid bacteria in starting their action on the fat. There is even a possibility that this small quantity of lipase may have been produced by udder bacteria. Palmer[309] and others claim that fresh milk contains no lipase. It is agreed, however, that there has been practically no hydrolytic cleavage of the fats in fresh milk due to milk enzyme. A purely chemical change in the fat takes place in the presence of oxygen and sunlight, when oxidation goes on at a slow rate. As a result the butterfat shows tallowiness in contrast to the rancidity produced by bacterial action. It is thought that bacteria may also, at times, play a part in the oxidation of the fat. It is possible that the bacteria may often be important in the first stages of oxidation and that their protoplasm may act as an acceptor in the process. Rahn[326] states that oxidation prepares the fat so that the bacteria, yeasts and molds will grow well on it.

The chief action of bacteria on the fats, however, is a hydrolytic cleavage by means of a lipase, in which the fats are split into glycerine and fatty acids. Glycerine is readily acted on by a number of bacteria and is usually the first of the split products to be attacked. The course of the fermentation of the glycerine will depend upon the organisms present and the general conditions, but it is generally changed to lactic acid, volatile fatty acids and aldehydes.[326] It is said[181] that a high proportion of unsaturated fatty acids, and especially of oleic acid, predisposes a mixture of fats to a tallowy oxidation in the presence of lipase. The double bond of oleic acid is regarded as a point of attack from which the products causing rancid odors arise. The fatty acids which result from the hydrolysis of the fats or from the fermentation of the glycerine are usually not attacked until the more available glycerine is destroyed. Then, however, these acids may be further split by some types of bacteria to simpler acids or to carbon dioxide and water. The first part, then, of the action of bacteria on fat is usually hydrolysis and this is followed by oxidation processes.

Aerobic conditions are considered necessary for the growth and activity of most fat-splitting microorganisms. Söhngen [391] has noted an exception in that certain bacteria can saponify milk fat anaerobically and can oxidize the by-products aerobically.

Since the action of bacterial lipase is inhibited by an acid reaction,[102] conditions which are unfavorable to an acid fermentation may help the decomposition of fat. Söhngen [390] has found that a temperature of from 10° to 15° favors fat-splitting in milk, and that this action goes on until acidity begins to develop. Organisms like *B. fluorescens liquefaciens, B. punctatum, B. lipolyticum,* and some of the micrococci may be active. He also found that some of the thermophilic bacteria could split fats, and that the lipase of some organisms was very heat resistant. It is possible, then, that the decomposition of fats by bacteria or their enzymes may take place at high temperatures.

It has been shown by many workers that some bacteria are able to synthesize fats as well as decompose them. Kluyver [226] has outlined a scheme for the synthesis of fats from carbohydrates and in accordance with his theories hydrogen transference reactions play a major role. Intermediate products are methyl glyoxal hydrate, pyruvic acid, glyceric aldehyde, glycerol, acetaldehyde, aldol and higher aldehydes. Bacteria are also able to form fat from proteins.

Stored fat has been noted in the bodies of many bacteria. The acid-fast bacteria are examples of organisms which elaborate a lipase and are known to have a particularly high fat content in their own cells. *Bact. pyocyaneus, Staph. aureus, Bact. prodigiosus* and other bacteria have been found to produce lipases which are able to synthesize esters of fatty acids.[486] A synthesizing action of bacterial lipase has been observed by Söhngen.[389] While the synthesis of fats by bacteria may be of considerable importance to the bacteria in their metabolic processes, it is unlikely to be of any importance in its effect on milk from the standpoint of human consumption.

A large number of fat-splitting bacteria have been found by various workers, but most of these organisms are not ordinarily important in milk. According to Gorini [184] some of the udder cocci can split butterfat and may play an important part in the ripening of certain types of cheese. Evans [112] has found *Bact. abortus var. lipolyticus* in considerable numbers in freshly drawn milk and says that they may hydrolyze fat within 24 hours with the production of disagreeable odors and flavors. The acid-forming bacteria in general do not elaborate a lipase, and their acid production usually inhibits the growth of lipase-forming organisms and the action of their lipase. A number of the actively proteolytic organisms of milk are also lipolytic [390] and under favorable conditions may split the butterfat in addition to their action on milk proteins. Thus the fatty acids from the fats would be present in addition to the undesirable protein split products.

Lipase has been found in: *Bact. pyocyaneus, Staphylococcus pyogenes aureus, B. prodigiosus, B. fluorescens, B. indicus, B. ruber,*[109] *Clado-*

sporium butyri,[300] *Bactridium lipolyticum,*[201] *B. Stützeri, B. punctatum,* certain spore-forming thermophilic rods,[390] *B. proteus, B. mesentericus,*[217] *Micrococcus tetragenus,*[372] *Staphylococcus albus,*[374] *B. putrificus,*[391] and other bacteria. Most of the molds can split fats, and Torulae [346] have been found active in this respect.

Conditions favorable for the splitting of butterfat by bacteria are more apt to be found in special milk products than in milk. The rancidity of butter is generally attributed to the action of microorganisms. Various bacteria, yeasts and molds have been deemed responsible for the rancidity. Butter which contains a relatively large amount of the aroma-producing constituent, biacetyl, is said to be more susceptible to the tallowy fat decomposition than butter lacking this constituent.[168, 419] Some kinds of cheese depend, in part, on the splitting of the fat by various organisms for their characteristic flavors.

Nitrogenous compounds as a source of carbon. Organisms will ordinarily use a fermentable carbohydrate instead of a protein as a source of energy, but it has been shown by a number of workers that under certain conditions nitrogenous organic compounds can serve as a source of both carbon and nitrogen for bacteria, and can be used for both energy and growth. Most of this work has been done with simple compounds like the amino acids. Some organisms are able to use certain amino acids as their only source of carbon for both energy and growth. Frazier and Rupp [120] found that of the amino acids, aspartic and glutamic acids served as the best combined source of nitrogen and carbon for certain of the proteolytic organisms of milk.

The decomposition of the various amino acids by bacteria has been reviewed by Hirsch.[184] He cites four typical reactions: (1) a decarboxylation from which an amine would result, (2) a reductive-deaminization which would give a fatty acid, (3) a combined carboxylation and deaminization, and (4) a combination of the third type with an oxidation process. The products of these reactions may be further broken down by bacteria to be used either as energy sources or for the building up of the cell.

The process of decomposition down to amino acids must take place by the action of the various proteolytic enzymes which are discussed under "Nitrogen metabolism in milk." When an organism breaks down considerable amounts of the milk proteins, it is assumed that the organism is probably using some of the protein decomposition products as a source of carbon for energy, and may be using some of the carbon for synthetic purposes.

Nitrogen metabolism in milk. The nitrogenous compounds of milk may serve as food for growth or for both growth and energy. Their use as an energy food has been discussed under carbon metabolism.

The form in which nitrogen is assimilated by the bacterial cell differs with the organism. Some workers think that the amino acids are the "building stones" from which the bacteria build up their cell proteins. Bacteria seem to prefer certain amino acids for their growth. Koser

and Rettger,[285] however, claim that the various amino acids are quite similar in their ability to support the growth of certain bacteria. These authors state that some organisms are apparently able to utilize amino acids directly without the aid of enzyme action. It seems reasonable to suppose as did Benton[80] that some bacteria may be able to use only a few of the amino acids while others may use any one of a number.

Some bacteria, like the alkali-formers of milk[12] can use nitrogen in still simpler forms like urea and ammonia. Organisms which can use ammonia as a sole source of nitrogen apparently can use any of the simpler amino acids if the medium contains a fermentable sugar as a source of carbon, according to Frazier and Rupp.[120]

Kluyver[226] has outlined a scheme for the synthesis of amino acids from sugar and ammonia in which a keto acid combines with ammonia to form a hydroxyamino acid which loses water to form an imino acid; the latter is reduced to an amino acid; the necessary energy is secured from an accompanying oxidation reaction involving a separate intermediate product of sugar decomposition. Virtanen[457] pointed out the synthesis of aspartic acid by coli bacteria and by *B. fluorescens liquefaciens* from fumaric acid and ammonia. It has been shown by Acklin[1] that *Bacterium pyocyaneum* can build up ammonia to alanine and then to the bacterial protein. Ehrlich[108] believed that organisms do not synthesize their body protein by condensing amino acids to polypeptides, but break down amino acids still further before synthesis takes place.

Some bacteria seem to be unable to use the amino acids, even a mixture of them, but require them in a combined form like a peptide or polypeptide or even in a more complex form. This is true of lactic acid bacteria, according to Orla-Jensen,[306a] Kluyver[227] and others.

In order for the bacteria to be able to use the milk proteins, then, they must split these proteins to simpler forms. Comparatively little is known about the proteolytic enzymes of bacteria, and the classification of these enzymes has been avoided. They are considered by some to be similar to the digestive enzymes of animals: pepsin, trypsin and erepsin, with rennin as the casein-coagulating enzyme. This idea is borne out by the fact that some bacteria can decompose a protein like casein and hence possess a pepsin-like or trypsin-like enzyme, while others must have protein degradation products as food and apparently have only an erepsin-like enzyme. Some milk organisms like those of the "acido-proteolytic group" of Gorini[182] secrete an enzyme which resembles pepsin in being proteolytic in an acid medium, although unlike pepsin its action is favored still more by a neutral reaction. Most proteolytic enzymes of bacteria, however, are more active in a neutral or alkaline medium and in this way resemble trypsin.

Waksman and Davidson[463] found that these microbial proteases may act both at a weakly acid and a weakly alkaline reaction. Wilson[477] found that protease production is a process of ordinary cell metabolism and is not dependent upon the ingredients of the medium except as these stimulate or inhibit cell growth. Many bacteria possess a rennin-like

enzyme which coagulates casein in a manner similar to the action of animal rennin.

It has been shown by Tarnenen,[420] Virtanen [455] and many others that proteolysis, like sugar decomposition, is not dependent upon the life of the microorganisms, but is, at least in many cases, a purely enzymatic degradation which continues when the cells die or are killed by treatment with toluol. The rate and extent of proteolysis is dependent on the reaction of the medium.

It is claimed by Diehl [99] that bacteria elaborate proteolytic enzymes to correspond to the different amino acids present in a medium and that the enzymes then attack these amino acids whether combined or free. He says that "the specificity of the proteolytic enzymes is resident in the amino acids composing the proteins and not in the proteins themselves." According to this viewpoint the amino acid would be a key by means of which the proper proteolytic enzyme would be able to unlock the protein molecule. This would lead to the supposition that the proteolytic enzymes of bacteria may differ considerably with variations in the organism and in the media.

The course of the decomposition of the proteins by bacterial proteolytic enzymes apparently resembles the action by animal enzymes.[376] The proteins go to proteoses, peptones, polypeptides, peptides and amino acids. In milk proteolytic bacteria cause an increase in non-protein nitrogen [123] and usually an increase in ammonia and amino nitrogen. Many bacteria possess enzymes which act on amino acids and produce, besides ammonia or amines, a variety of products, depending upon the amino acid.

It has been shown by Bainbridge [16] and by Sperry and Rettger [395] that bacteria which can split proteins can not break down purified proteins or proteoses [82] unless small amounts of simpler nitrogenous food are available to get them started. According to these workers some bacteria are unable to split proteins or proteoses under any condition and must have simpler nitrogenous compounds as food.

Milk contains as a possible source of nitrogen simple nitrogenous compounds, casein, lactalbumin and lactoglobulin; after bacterial action has started, there will also be present split-products from the original nitrogenous constituents.

The simple nitrogenous compounds as a source of nitrogen. Various workers claim to have found in fresh milk small amounts of lecithin, cephalin, urea, uric acid, creatine, creatinine, amino acids and ammonia. (See Chapter I.) Certain bacteria can readily use some of these substances while other bacteria can use none of them. Since very small amounts of nitrogen are necessary for bacterial growth, these simple compounds may support considerable growth in some cases. They may also serve to give certain caseolytic bacteria a start so that the casein can be attacked.

Frazier and Rupp [120] found that certain milk bacteria were able to ferment urea and use it as a sole source of nitrogen in a synthetic medium, and apparently were able to break down the urea in milk with the forma-

tion of ammonia.[128] No reports have been found on the action of bacteria on other simple nitrogenous compounds in milk or on the disappearance of these compounds from milk. It is known, however, that bacteria, which can not start to grow in a medium containing purified casein without the addition of simpler nitrogenous compounds, can grow well in milk.

Casein as a source of nitrogen. Casein is the chief nitrogenous compound of milk and a study of nitrogen metabolism in milk is concerned chiefly with its decomposition. The decomposition of casein by bacteria may range from an undetectable change to a practically complete destruction. The degree and kind of decomposition may not only vary with different kinds of organisms, but may vary considerably under different conditions with the same organism.

According to their action on casein milk bacteria may be roughly divided into four groups: (1) those which can not split casein, (2) those which attack casein only as a source of nitrogen, (3) those which under some conditions use casein only as a source of nitrogen and under other conditions attack casein as a source of both nitrogen and energy, and (4) those which use casein as a source of both nitrogen and energy. Most of the important milk bacteria fall into one of these groups, although a few organisms may seem hard to place. It is possible, of course, that organisms which break down little casein and are apparently using the casein only as a source of nitrogen may be also obtaining carbon compounds for growth purposes from the same source.

Proteolysis by the true lactics. The "true lactics" are not considered proteolytic bacteria. Organisms of the *Streptococcus lactis* group can use casein and break it down to peptones [278] or amino acids,[6, 24] but do not ordinarily produce enough change in the casein to be detected chemically. It is only after the addition of an excess of calcium carbonate and an incubation period of two or three months that a measurable increase in amino nitrogen is found.[24] Hammer and associates [6, 165] have found that those strains of *S. lactis* which coagulate milk the most rapidly are the most proteolytic. A temperature of 14° to 16° favors the caseolytic action of organisms like *S. lactis* and this action decreases with a rise in temperature.[22] While *S. lactis* can attack casein it seems to prefer simpler nitrogenous compounds. Consequently the associative action of an actively proteolytic organism like *B. subtilis* [265, 319] or *Pseudomonas fluorescens* [258] assists its growth in milk. Virtanen [446] has found that *S. lactis* and *Bact. casei* ε together decompose casein better than either alone. According to Orla-Jensen [308] true lactics can not use ammonia or simple amino acids as a nitrogen source.

In addition to *S. lactis,* Orla-Jensen lists among the "true lactics" which are proteolytic on casein: *S. cremoris, S. bovis* and *S. liquefaciens.* Cocci, isolated by Eagles and Sadler [106] from Kingston cheese and classed as true lactics, were found caseolytic. Sanders and Frazier [366] grew *S. thermophilus* for one month at 29° in milk-chalk mixtures and found that casein was not attacked. Knudsen [280] found that some strains of

S. cremoris are strongly caseolytic and others are not, while Barthel found the organism non-proteolytic.

The lactobacilli of which *L. bulgaricus* (the *Bakterium casei* ϵ of Freudenreich) is a representative are also considered true lactics. Like the *S. lactis* group they produce little change in the casein over short periods of incubation; but after several months in a neutral or alkaline milk a measurable decomposition takes place.[446] Freudenreich divided the lactobacilli of cheese into four groups which he called *Bakterium casei* α, γ, δ and ϵ.[459] Of these the α and ϵ type can attack casein after a long incubation with the direct production of amino acids and ammonia,[308] while the γ and δ types do not attack casein. *B. casei* ϵ organisms have been shown to digest casein, even after the cells have been killed with toluol.[420, 455] An autolyzed enzyme preparation from the cells apparently contained two enzymes, a proteinase and a polypeptidase. These organisms are proteolytic at a higher temperature than is *S. lactis*.

The action of the true lactics on the casein seems to be due to an endoenzyme which does not act on the casein until after the death and disintegration of the bacterial cell. Since the action takes such a long time, it is not important except in the case of cheese ripening where several months are allowed for the action. The action of the true lactics on casein places them in the group of bacteria which apparently attack the casein in milk only as a source of nitrogen.

Proteolysis by the colon-aerogenes group. Organisms of the colon-aerogenes group of "pseudo-lactics" vary in their action on casein. Weigmann[469] says that *B. aerogenes* can not attack casein, while *Bact. coli* and similar organisms can do so. He says that the amount and kind of decomposition of the casein will depend on the quantity of acid produced by the organism. In general an organism which produces less acid will cause more protein decomposition. In the case of weak acid formation the protein decomposition may go on to such an extent that an alkaline reaction results. According to Weigmann this action is aided by an erepsin so that casein is broken down not only to amino acids but to still simpler compounds.

Since the amount and kind of protein decomposition depends on the organism and on conditions in the medium, reports by various workers on the action of *E. coli* differ considerably. Kendall, Day and Walker[215] state that *B. coli* and *Bact. cloacae* have little action on the proteins of milk. Staffe[401] claims that *B. coli* produced proteolysis in milk held at 30° for seven weeks, but Zaribnicky[488] states that *B. coli* is non-caseolytic in milk. Swiatopelk-Zawadski[417] found that *B. coli commune* was one of the most actively proteolytic of the common aerobic milk organisms. Taylor[425] says that *B. coli communis* digests casein mainly into proteoses and peptones with the formation of only a small percentage of amino acids. Indol may be one of the simpler nitrogenous by-products although it may not be formed in milk.

Between *E. coli* and *A. aerogenes,* organisms of this group are found whose action on milk takes in varying degrees of difference between these

two representative species. *B. ichthyosmius*[400] has been found to cause marked splitting of the protein in butter.

It is to be expected that, because of the acid produced, the growth of *S. lactis* or *L. bulgaricus* along with *E. coli* decreases casein decomposition by the latter.[28]

The colon-aerogenes bacteria can be divided on the basis of their action on casein into the aerogenes-like organisms which attack the casein not at all or in undetectable amounts, and the coli-like organisms which under some conditions apparently use the casein only as a source of nitrogen and under other conditions seem to use some of the casein for both nitrogen and energy.

Proteolysis by acid- and rennet-forming cocci and rods. The acid- and rennet-forming cocci include most of the udder cocci[174] and some cocci from other sources. These organisms vary considerably in their proteolytic power.[469] Some produce acid-rennet curd which they redissolve; others apparently decompose the casein to a lesser extent. While for most of them the optimum temperature for growth and acid production is near blood heat, they usually show more proteolytic action at lower temperatures where less acid is formed. The organisms which are proteolytic in an acid reaction are called by Gorini "acido-proteolytic" and are classed as "Tetracocci" by Orla-Jensen. The *Micrococcus casei liquefaciens* of Freudenreich is one of the more active proteolytic organisms of this type.[301] Casein is decomposed with the production of large quantities of albumoses and peptones with less amino acids and ammonia.

Most of these cocci ferment lactose, usually with enough acid to curdle the milk. But the presence of the fermentable carbohydrate, lactose, does not seem to influence greatly the amount of protein decomposition. Many of the cocci produce a yellow to orange pigment. Numbered among the yellow cocci which can decompose casein are *M. perflavus, M. conglomeratus, M. subflorescens, M. varians, M. cereus* and *M. citreus* (Hucker). Among the non-chromogenic cocci are *Staphylococcus albus, M. freudenreichii* and *Tetracoccus liquefaciens* (Orla-Jensen).[119, 123] Gorini has named his acido-proteolytic cocci: *Caseococcus, Mammococcus, Gastrococcus* and *Enterococcus*.

These proteolytic cocci, then, under some conditions decompose little of the casein and seem to use it chiefly as a source of nitrogen, while under other conditions larger quantities of casein are broken down; and energy is probably obtained from some of the nitrogenous by-products. The enzymes of most acido-proteolytic bacteria are said by Gorini[186] to be very active at pH 4.8 to 5.1 and to consist of proteinases and peptidases.

There are a few acido-proteolytic rod-shaped organisms; but they are usually uncommon in milk unless it has been held for some time at low temperatures. Bacilli of this type have been reported by Gorini,[182] Sandelin,[365] and others.

Proteolysis by other non-spore-forming rods. Of the remaining non-spore-forming rods some of the *Proteus, Achromobacter, Flavo-*

bacterium and *Pseudomonas* genera, including alkali-formers and fluorescent bacteria are proteolytic in milk. Many of these bacteria are important in causing changes in milk held at low temperatures.

The *Proteus* group contains some bacteria which peptonize milk rapidly and others which apparently do not attack casein. Organisms like *Proteus vulgaris* break down the casein rapidly by means of a trypsin-like enzyme [218] with the production of relatively small amounts of amino acids and ammonia. Milk is first made a little acid by *Proteus vulgaris,* due to its action on the casein [215] and then becomes alkaline as the action continues.

The alkali-forming bacteria of milk consist of both cocci and rods; and some of the rods, like *Alcaligines albus* [31] and *Alcaligines bookeri,*[119] are able to peptonize milk. Many of the fluorescent bacteria of which *Pseudomonas aeruginosa* (*B. pyocyaneus*) is a representative, are actively proteolytic and rapidly peptonize milk by means of a trypsin-like enzyme. *Ps. aeruginosa* produces a transient acidity in milk and then alkalinity. Another member of the group, *Pseudomonas fluorescens,* apparently has no trypsin but only an erepsin [462] and attacks only split-products of the casein decomposition started by other organisms. Some of these organisms also possess a rennin-like enzyme.

Achromobacter liquefaciens, A. delictatulum and *Flavobacterium synxanthum* [119] have been shown to be proteolytic in milk.

Virtanen [445] found that his propionic acid bacteria slowly decomposed casein when they were grown in milk with calcium carbonate at 37° for two months. The proteus, alkali-forming and fluorescent bacteria which decompose casein usually break down large amounts and apparently use the protein as a source of both nitrogen and energy.

Proteolysis by spore-forming rods. The majority of the actively caseolytic bacteria of milk are spore-forming rods, and these are chiefly aerobic or facultative organisms of the subtilis-mesentericus group. Ford et al [246, 247] found *B. cereus, B. subtilis, B. albolactis* and *B. vulgatus* to be most common in the milk which they examined. Similar results were obtained by Frazier and Rupp [119] who found *B. albolactis* and *B. cereus* most common, but also isolated *B. vulgatus, B. subtilis, B. simplex, B. mesentericus, B. cohaerens, B. tumescens, B. megatherium, B. ruminatus* and *B. macerans* from milk. These organisms were found to fall into three groups according to the results of their action on milk: (1) high acid, low amino nitrogen, (2) low acid, low amino nitrogen and (3) low acid, high amino nitrogen.

These proteolytic spore-forming rods are found in most samples of milk, but predominate and cause deep-seated changes only under certain conditions. Ordinarily acid-forming organisms like *S. lactis* will predominate if originally present in good numbers, but the growth of these acid-formers may be assisted by the proteolytic action of associated spore-forming rods. When the acid formers are few in number, weak or absent, the proteolytic bacteria may predominate and break down the milk proteins to a considerable extent. This is particularly apt to be true in

pasteurized or boiled milk where spore-forming rods may be the only surviving bacteria.

The action of the proteolytic spore-forming rods on the milk proteins varies with the different organisms. Most of them have a curdling enzyme, rennin. Some coagulate the milk with this enzyme before splitting the casein and some break down the casein without any sign of curdling. This same variation may be found from time to time with a pure culture of a given organism. It has been noted by Gorini [188] and by Conn [71] that some organisms, which curdle milk with a rennin-like enzyme when first isolated, lose that property on continued cultivation and have increased proteolytic power. It is supposed that the proteolytic action becomes so rapid that the action of the rennin is not evident. This may account for the lack of agreement among writers as to the ability of certain organisms to sweet-curdle milk before they digest it. A few of the organisms, like *B. albolactis,* can ferment lactose and form a shrunken acid-rennin curd with considerable expressed whey. This curd is later digested.

A neutral or slightly alkaline reaction is most favorable to the organisms and most of them tend to produce an alkaline reaction in milk. *B. subtilis,* for instance, produces a gradually increasing alkalinity in milk.[216] Organisms like *B. mycoides* [469] first make the milk weakly acid and then rapidly break down the casein and produce an alkaline reaction. *B. mesentericus* while decomposing the casein produces an increasingly acid reaction in milk [216] although it does not ferment the lactose. Most of the organisms are not active in fermenting sugars and most of them can not attack lactose, with the exception of a few organisms like *B. albolactis.*

If enough time has been allowed for action, most of these actively proteolytic organisms are able to decompose the greater part of the casein and a more or less clear amber or brown liquid usually results. Some of the casein is broken down as far as amino acids and ammonia, while some of the nitrogen is left in the form of proteoses, peptones and peptides. At any stage in the decomposition all of the forms of nitrogen are apparently undergoing a change and no one form seems to be completely destroyed.[344]

Most of these proteolytic spore-forming rods apparently use the milk proteins as a source of food for energy and growth. Not only is nitrogen for growth obtained but also carbon.

Proteolysis by the spore-forming anaerobic rods. The various spore-forming anaerobic bacteria which may be found in milk show different ability to break down milk proteins. These differences range from very actively proteolytic organisms like *Clostridium putrificum* to organisms like *Clostridium butyricum* which has little or no action.[469] *C. putrificum* under favorable conditions breaks down the milk casein to a greater extent than the other organisms and produces the odor of putrefaction that gives it its name. Practically all of the casein is broken down, and much of the nitrogen is carried to very simple compounds.

Clostridium sporogenes and *Clostridium welchii* are both proteolytic according to Wolf,[484] but *C. sporogenes* is more active than *C. welchii*. *C. welchii* is considered actively proteolytic by Sears [377] but is called primarily fermentative by Rettger and Newell.[333]

Proteolysis by other milk bacteria. The remaining milk bacteria are for the most part non-proteolytic. Most of the thermophilic and thermotolerant bacteria fall into one of the classes discussed above. Organisms of the spore-forming aerobic type are usually actively proteolytic. The occasional inert types of bacteria which enter milk from various sources are not of enough importance in milk to be discussed.

Lactalbumin and lactoglobulin as food for energy or growth. Little work has been reported on the action of milk bacteria on lactalbumin and lactoglobulin. In a preliminary report Supplee [412] concluded that some species of bacteria could attack the lactalbumin in milk more readily than the casein. Among the organisms which caused a decrease in the albumin were: *Mic. albidus, Ps. liquefaciens, Bact. bulgaricum, B. coli communis* and *Bact. aerogenes*. Frazier and Rupp [123] found that certain organisms which do not attack casein in milk are able to decompose lactalbumin, and that most of the milk organisms which can attack casein can also decompose lactalbumin. *S. lactis, L. bulgaricus* and *L. casei* were apparently able to decompose lactalbumin. Some of the proteolytic cocci did not decompose as great a proportion of albumin in milk serum as they did of casein in milk. Kieferle and Gloetzl [221] report that when milk sours naturally the albumin is decomposed more rapidly than the casein.

Oxygen Requirements and Reducing Abilities of Bacteria

It is well known that, under ordinary procedures of artificial culture, certain species of bacteria grow best when the entrance of oxygen is excluded, others grow best when an abundance of oxygen is supplied, others are more or less indifferent so far as mere growth is concerned and still others seem to require conditions suggesting the beneficial effects of partial oxygen tension. No fine spun objections to the terminology employed in the description of the more prominent facts of these phenomena, and no difficulties in the scientific analysis of the conditions imposed in specific cases need stand in the way of certain practical applications of the observed facts.

Historical development. The first definite observation of anaerobiosis we owe to Spallanzani, but it was the observation of Pasteur upon the effect of air on the organism now known as *Clostridium butyricum*, accompanied by the use of various and sundry anaerobic methods for the isolation of new species by Pasteur and his pupils that gave impetus to the study of anaerobiosis. It was of course obvious to Pasteur and his coworkers that there are innumerable ways of accomplishing the conditions of anaerobiosis and the greater number of the devices employed today were employed *in principle* in Pasteur's laboratory. Exhaustion by the pump, displacement with other gases including steam, chemical

absorption, delay in the penetration of oxygen by various barriers, biochemical utilization of residual oxygen by symbiosis, etc., permitted the early experimenters to culture organisms resistant to open-air methods. It is hardly worth while to do more than mention two reviews[198, 394] in which descriptions of various devices and principles may be found.

It was not long after the earlier methods were put to use that Gunning[144] contested the interpretations that bacteria can grow in the complete absence of oxygen. The essence of his contention was doubt of the perfection of oxygen-removal, and the claim that in his experiments growth was the more inhibited the nearer his media approached complete removal. It was the beginning of a long controversy. In this the points at issue were not always those raised by Gunning but were concerned with a wide variety of observational facts. For instance, Beijerinck,[26] on the basis of most interesting experiments dealing with the orientation of bacterial growths about plant cells, etc., was led to the conclusion that all bacteria require some oxygen at least some time in their life history. He even went so far as to say that the requirements were too minute to be detected by chemical means.

These and minor observations, and conclusions from the observations, started an era of fine spun nomenclature. Beijerinck divided bacteria into aerophils and microaerophils. This philosophy is traced in the contradictory term "aerotrophic anaerobe" found later in the literature of pathology. The meticulous preferred oxybiosis to aerobiosis and anoxybiosis to anaerobiosis. There were employed such terms as temporary and pseudo aerobes, partial tension aerobes, microaerobes, oxygenophyles, aero-anaerobes, paraoxygenophyles, prosaerotactic organisms. Each had its origin in an attempt to describe some delicate adjustment, the observed fact underlying which must some day be accounted for.

In the meantime all sorts of variation in cultural conditions were being tried. There occurred for instance a long series of papers dealing with the culture of "anaerobes" in media exposed to the air but in the presence of fresh vegetable or animal tissue. Parallel with this were experiments dealing with the symbiotic aspect of aerobe-anaerobe associations, some of which are remarkable in showing that so-called strict anaerobes may develop in the presence of aerobes while oxygen bubbles through the medium.

But then came the era of the study of "vitamins," "hormone media"; the study of various physical properties of cultures, enhancement of growth by CO_2, special food requirements, the accumulation of H_2O_2, mass effects, inhibitory and enhancing principles elaborated by one organism in relation to the growth of another.

Present status. It is [illegible] realized that the interpretation of some of the data in the vast litera[illegible] the subject is not an easy task. There is, however, one means of [illegible] the problem anew.

All cultures are in [illegible]nce of water. The energy of decomposition of water is kn[illegible] the intensity factor of this energy is expressed in terms of [illegible] potential it is known to be about 1.23

volts. Adopting the hydrogen electrode potential difference at one atmosphere of hydrogen and one normal hydrion concentration as an arbitrary zero, it may be said that any system in a solution normal with respect to the hydrogen ions, which has a reducing intensity sufficient to support one atmosphere of hydrogen, should yield an electrode potential of zero; and any system in equilibrium with one atmosphere of oxygen should have a potential of 1.23 volts. This "spread" remains as the pH value of the solution increases but the individual values become more negative by approximately 0.06 volts per unit of pH increase.

Now it was shown by the investigations of Gillespie;[129] Clark;[65, 67] Clark, Cohen and Gibbs;[68] Cannan, Cohen and Clark;[56] and Clark and Cohen,[69] not only that bacterial cultures give directly electrode potentials indicative of such high reducing potentials that they may even approach or overstep the potential of the hydrogen electrode, but that the dyes they reduce have comparatively highly negative equilibrium potentials. From such potentials there can be calculated[66] the theoretical pressures of oxygen which would have to occur *if the dye systems in question attained equilibrium with oxygen.* While it has never been *demonstrated* that such an equilibrium has been attained in culture there is no reasonable doubt that there have occurred circumstances most favorable to this attainment. If so there remains not the slightest doubt that the reduction of free oxygen to a point where it can have no significance for energy changes and can have no function in the sense of a vitamin has been attained. The calculated partial pressure of oxygen in some of these cases would be such that one discrete oxygen molecule would be left in a volume the edge of which is one million meters long—a "calculation-value" the real meaning of which is merely complete absence of free oxygen.

On the other hand several lines of experiment are now converging to the conclusion that the participation of oxygen in the processes of life is seldom a question of equilibrium states. This explains, of course, the lack of any significance in attempts to arrive at quantitative data by imposing definite partial pressures of oxygen without taking into consideration variations in rates of reaction. This variability in rate is introduced whenever anaerobes are studied in the presence of materials biological or otherwise which themselves are capable of taking up oxygen or are capable of acting in any way upon the catalysis of oxygenation.

In short the problem has been resolved into two distinct aspects. Wherever true equilibrium conditions are concerned we are prepared to deal with the matter in an exact and definite manner provided the reactions concerned are directly or indirectly connected with reversible reactions of the type of methylene blue-leuco methylene blue, and indigo-indigo white systems which have played a great part in furnishing the data of anaerobiosis. But concerning these varied conditions in which rates of oxygenation are concerned little can be done with the methods available. In view of this situation it now remains to be seen how many of the facts which have entered the subject can be dealt with in terms of the reducing abilities of bacteria.

Reducing abilities of bacteria. Although adequate quantitative investigation of the reducing abilities of bacteria has been forced to await the development of physico-chemical technic, empirical observations long ago suggested certain tests which actually depend upon rapidity and intensity of bacterial action. Litmus is not limited in its usefulness to the indication of acidity—it is characteristic of some organisms to reduce it to its colorless form, the rapidity of the reduction depending on the species. For example, a tube of litmus milk inoculated with *Escherichia coli* and incubated at a temperature favorable to growth will reduce the litmus, but not until several hours after acidity is evident. With *Streptococcus lactis,* on the contrary, reduction of the litmus is very prompt, and occurs before the acidity is sufficient to turn the litmus pink or to coagulate the casein. An equally vigorous reduction is observed with methylene blue. Sherman and Albus [378] were able to distinguish *S. lactis* from the pyogenic streptococci by this reaction. Janus green has been used as a substitute for methylene blue, and Mundinger and Wolf [279, 280] have recently introduced the use of resazurin and azorufin as oxidation-reduction indicators for milk.

Methylene blue test. The methylene blue or reductase test has been used for years as a test of quality in milk. It is of great value as a method of estimating roughly the progress of deterioration due to bacterial multiplication. The differences of opinion as to its reliability and the somewhat inconsistent findings of workers in different places, can be cleared up by a more thorough understanding of the state of affairs which is recorded by the progressive bleaching of the indicator. Fresh milk, freed of dissolved oxygen, and held in contact with methylene blue under strictly anaerobic conditions will reduce it, usually within 2 hours. Under ordinary circumstances this reaction is opposed by the free oxygen present, and does not occur until the natural reducing power of the milk is reinforced by bacterial action. Barthel [28] suggested that the reductase test involved two processes: first, exhaustion of the dissolved oxygen by bacteria, which permits the natural reductase of the milk to function; and second, the reduction of the dye by constituents of the milk.

Mechanism of reduction by bacteria. Decolorization of methylene blue does not result from a removal of oxygen from the molecule, but rather from the addition of hydrogen. The actual mechanism of bacterial reduction has been and still is the subject of argument. When an inoculation is made on the surface of agar impregnated with methylene blue, the destruction of the color is not confined to the vicinity of the colonies.[396] Such observations suggested the idea that the active agent was some diffusible product of metabolism or exoenzyme. Attempts to isolate such a reductase from cultures by filtration or other means have been fruitless. This is not surprising in view of the lability of the methylene blue test with respect to low degrees of heat and weak antiseptics,[176] which fact eliminates enzymes from consideration and suggests that the reduction of the dye is intimately associated with vital cell processes. Fred [126] observed partial reduction in a solution of methylene blue, suspended in a collodion

sac immersed in milk. However, the reaction proceeded much more slowly than when the stain was added to the milk directly. Likewise, old milk cultures, in which further multiplication was inhibited by a minimum effective dose of toluol, showed a very slow reduction. Fred concluded that methylene blue reduction by bacteria must be both intra- and extra-cellular, and suggested that during bacterial assimilation nascent hydrogen might be given off.

Gorzoni and Kramar [189] point out that after the maximum population has been attained in a milk sample, the reducing power falls off progressively during the period of decrease in the number of living cells. They concluded that reduction by bacteria is a truly vital phenomenon and intimately connected with respiration. Oberstadt [296] in 1913 stated as his opinion that the reduction processes take place outside the cell, but in contact with it, as a secondary phenomenon in the oxidation of sugars and other nitrogen-free substances entering into bacterial metabolism. In this connection, and in considering Thunberg's [482] work on metabolites, it must be borne in mind that any reduction implies inevitably the oxidation of some other substance. This oxidation may perhaps be a reversible one, in the case of some of the metabolites, or it may be irreversible as in the fermentation of sugar. A very vivid and suggestive picture of this process as taking place in areas of intense reduction-potential on the cell surface is given by Quastel [323] in an article correlating findings on bacterial oxidations and reductions with the Wieland hypothesis of fermentation. Perhaps the most plausible theory for the mechanism of methylene blue reduction in milk was advanced by Thornton and Hastings.[481] This theory presupposes that decolorization of the dye involves a transference of hydrogen to the methylene blue molecule. The source of the hydrogen is presumed to be certain milk metabolites, of which sulfhydryl compounds, citrates, succinates and aldehydes may be considered examples. The dissolved molecular oxygen in milk is concerned in the maintenance of a certain positive oxidation-reduction potential. According to the theory of Wieland, some of the dissolved oxygen in milk is reduced by the hydrogen to H_2O_2 and H_2O. As bacteria grow in milk they also decrease the concentration of oxygen and this consumption of oxygen permits a drift of the oxidation-reduction to the negative side of zero. In the more positive zones of potential, oxygen has a greater affinity for hydrogen than does methylene blue, but as the potential becomes more negative a point is reached at which the reverse is true and the hydrogen reduces the dye.

Intensity of reduction. The methylene blue test, which is an indicator of a definite amount of bacterial activity in milk under definitely prescribed conditions, should not be expected to correlate closely with the plate count, which gives information as to the number of clumps of bacteria in a small portion of the milk which will grow on a given medium. This has been pointed out by Thornton and Hastings [481] who believe that both tests should be used in milk control work, one to supplement the other.

One important cause of discrepancies in the methylene blue test is the

fact that in some species the reducing activity is much more intense than in others. The relation of reduction time to numbers present would then depend to some degree on the nature of the predominant flora.[126] Fred inoculated sterile milk containing a known amount of methylene blue with 24-hour pure cultures and followed the reduction quantitatively by titanium chloride titrations. Of 22 organisms often found in milk, 21 showed marked and consistent reducing power. He pointed out that the time-reduction curves were logarithmic and closely resembled those observed and calculated for bacterial multiplication. Different species showed characteristic differences in the length of the lag period and in the steepness of the slope of the curve after lag was over. Such a curve records the quantity of dye reduced and is comparable to a titratable acidity curve in the study of acid formation. It can not tell the whole story. The apparent lag period is not necessarily identical with the lag period in the actual growth curve—it represents the time required for the culture to attain the intensity level at which the indicator is attacked. This level varies slightly with pH, and will consequently depend to some extent on the fermentative powers of the strain studied. The steepness of the slope is determined by the rapidity of multiplication in the medium, and the reduction intensity of the individual cells.

Until recent times the decolorization of the dye in the methylene blue test was brought about largely by *Streptococcus lactis* and closely related organisms. The present more stringent methods of milk production and control often result in the exclusion, or marked reduction of lactics in the milk. Under these conditions the decolorization of the dye is usually produced by udder streptococci, members of the colon-aerogenes group, and other types.

Reduction potential. The intensity factor in actual reduction-potential, is as important as the quantity factor and of equal, if not greater, significance. Gillespie[129] published the first time-potential curves for bacteria. They covered an enormous range of potential, only a small fraction of which can be observed by the use of any one indicator. Cannan, Cohen and Clark[56] have published a chart comparing the time-potential curves of four common organisms growing in milk at 37°. This is reproduced as Figure 35.

While the curves are not directly comparable, due to the very different changes in pH induced by the different species, it is evident that great differences are to be expected in the time required to reduce a particular indicator, such as methylene blue, whose potential-range at the pH of fresh milk is indicated by the wedge at the right of the chart. Later work by Frazier and Whittier[121, 122] with milk organisms emphasized the fact further that different bacteria run characteristic potential-time curves in milk and reach different levels of reducing intensity. With many of the organisms studied it was observed that the end of the rapid drop in potential was almost coincident with the beginning of the rapid increase in numbers of bacteria. With some organisms, however, like *Streptococcus fecalis, S. thermophilus* and *S. mastitidis*, it was necessary to have

large numbers of actively growing organisms in order to bring the potential to its most negative value. When two species of milk bacteria were grown together in sterile milk, in most instances one species largely controlled the Eh changes and produced characteristic alterations in the form of the potential-time curves of pure cultures. *E. coli* and other coliform organisms, when grown with *S. lactis* exerted a restraining influence on the rapid drop in Eh usually caused by pure cultures of *S. lactis*. *S. lactis* controlled the Eh changes during the first part of growth when grown

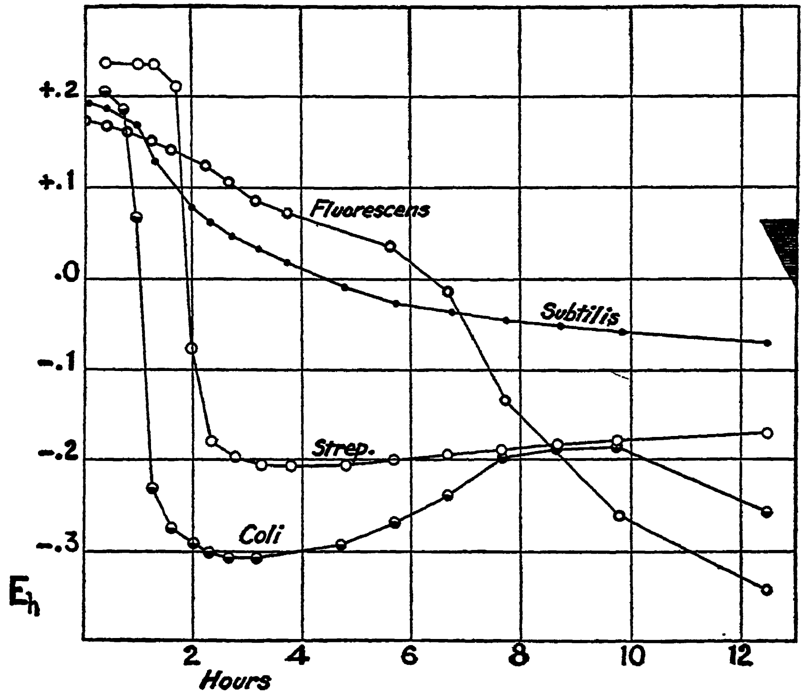

FIG. 35.—Comparison of time: potential curves of *Bact. coli, S. lactis, B. subtilis,* and *B. fluorescens* in milk at 37° C.

with *C. welchii,* but the latter organism brought about a more negative final value.

Reduction capacity. It is apparent from the experiences of several workers that the capacity factor of the oxidation-reduction systems of deoxygenated milk is small. Bubbling oxygen or air through milk which has been deoxygenated by *S. lactis* and brought to a potential of the order of —0.20 volts will cause the potential to rise immediately to the positive value characteristic of fresh milk. The value will then fall somewhat slowly to an intermediate value determined by the activity of the organism on the one hand and the rate of bubbling of the air on the other. Different bacterial species vary in their ability to combat the effect of oxygen on the potential value.[121] Evidence is accumulating which may shortly bring to

us an evaluation of the relative intensities and capacities of the several oxidation-reduction systems known to be present in milk.

Phases of Growth

Growth curves. A bacterial growth curve is a graphic presentation of the bacterial cell population in a limited volume of nutrient medium from the time of inoculation until the culture contains no living cells. However, the growth of a bacterial culture is seldom followed beyond the time required for the development of what is termed a "mature culture," which is approximately twenty-four hours.

The bacterial growth curve is of the same nature as the curves obtained by Robertson [343] on the growth of individual animals, by Reed and Holland [331] on the growth of plants, by Pearl [313] on the growth of colonies of fruit flies, and by Pearl and Reed [312] on human populations and is apparently subject to the same biological laws.

Possibly the first study of the growth of bacteria was that of Buchner, Longard, and Riedlin [58] in 1887 who calculated the generation time of *Vibrio cholerae* on plate cultures. Following this early work there has been a steady accumulation of data relating to bacterial growth curves. In comparatively recent years works of a biological and physiological nature have added greatly to our knowledge of bacterial growth and have given a greater significance to bacterial growth curves. In possibly no other field is an intimate knowledge of the factors involved in bacterial growth more rapidly applicable than to the handling of milk and the manufacture of dairy products.

When a nutrient medium is inoculated from a mature parent culture there follows a period in which there is no cell division, termed the "lag" period. Following this latent period the cells begin to divide and reproduction proceeds at approximately a logarithmic rate until the maximum cell population is approached. There is then a sharp breaking off in the curve and multiplication proceeds much more slowly until the maximum population is reached. The culture then becomes senescent. Whether reproduction takes place slowly, or at all, during this period of senescence, has not been definitely shown. At any rate the cell population remains practically constant during this period until the death phase begins.

According to Buchanan,[51] the growth curve may be divided into seven phases: (1) the initial stationary phase, during which the number of bacteria remains constant, (2) the lag phase, during which the average rate of increase in numbers increases with the time, (3) the logarithmic growth phase, during which the rate of increase per organism remains constant, (4) the phase of negative growth acceleration, during which the rate of growth per organism decreases, (5) the maximum stationary phase, during which there is practically no increase in the numbers of bacteria, (6) the phase of accelerated death, during which the number of bacteria decreases slowly at first and then with increasing rapidity and, (7) the so-called logarithmic death phase, during which the rate of death

per organism remains approximately constant. By some workers the growth curve is simplified by division into only four phases: the lag phase, which includes phases (1) and (2) of Buchanan, the logarithmic phase, the resting phase, which includes phases (4) and (5) above, and the death phase, which includes phases (6) and (7) above.

There have been numerous studies of the rate of multiplication of various organisms. Mathematical formulae have been evolved for the calculation of the generation time of bacteria and for the cell population of a culture, but these formulae are not generally applicable. McKendrick and Pai [260] made a mathematical study of the rate of multiplication of bacteria. They concluded that multiplication is in proportion to the number of cells and that the available food materials diminish in the same proportion. Robertson [348] interprets McKendrick's work as evidence that cellular multiplication is autocatalyzed in unicellular organisms as well as in plants and animals.

Morphological changes. The transitions of a cell from a period of reproductive latency through the successive stages of rejuvenation, reproduction, and then back again to a resting cell have been found to be accompanied by distinct morphological and physiological changes. Clark and Ruehl [68] have observed that, with exception of cells of the diphtheria group, the young cells are considerably larger than the older ones. Henrici [182, 183] has made an elaborate quantitative study of the morphological characters of a number of types of bacteria. He says, "The cells of bacteria undergo a regular metamorphosis during the growth of a culture, similar to the metamorphosis exhibited by the cells of a multicellular organism during its development, each species presenting three types of cells, a young form, an adult form and a senescent form, and that these changes are dependent on the metabolic rate, . . . the changes from one type to another occurring at the points of inflection in the growth curve." By making parallel measurements of cells and cell counts he found that the change in size "is definitely correlated with the rate of growth."

Physiological changes. Of equal interest, and of greater practical importance, are the physiological differences of young and old bacterial cells. Sherman and Albus [380] have shown that the newly formed bacterial cells of *Bacterium coli* and *Proteus vulgaris* show definite physiological characteristics which differentiate them from mature bacterial cells in that the young cells are more sensitive to the influence of their environment than are the mature cells. Cultures taken during the period of rapid growth were found to be sensitive to such mild hazards as brief exposures to relatively low heat, cold, 2 per cent NaCl, and 0.5 per cent phenol. Mature cells of the same organism were not materially affected by equal exposures to the same deleterious agents.

The practical applications of these findings to many of the problems peculiar to milk and its products become apparent. The errors encountered in the plate count of milk may be influenced, depending upon whether the bacteria in the sample to be examined are in the rapidly growing stage or are mature cells. In the case of samples held in ice—if the samples

were taken during the period of rapid growth, and suddenly exposed to such a low temperature, the count obtained would not indicate the true bacterial content of the milk from which the sample was taken because there would be a marked mortality of the young cells so exposed. The susceptibility of young cells is a factor to be reckoned with in a consideration of the relative merits of direct and cultural enumeration of bacteria.

The observations of Wilson [478] are of particular interest in connection with the direct enumeration of bacteria. Under carefully controlled conditions he demonstrated that even during the logarithmic phase the percentage of viable organisms seldom exceeds 90 per cent of the total number of organisms in a culture.

There can be little doubt that in the efficiency of the pasteurization of milk the sensitiveness of young cells plays an important part. This has been shown by Sherman, Stark and Stark [385, 402] in experiments on ropy milk bacteria and other organisms. Ayers and Johnson [11] have shown that the efficiency of pasteurization can not be judged on the basis of the bacteria destroyed. They found that in milks of high bacterial counts it is easy to obtain an efficiency of 99 per cent, while in milks of low bacterial counts no such efficiency of bacterial destruction is obtained. In milks of high bacterial counts, due consideration being given to the predominant types of bacteria, there must necessarily have been an extensive bacterial growth, while in the milks of low bacterial counts there has been little or no proliferation of cells. In the latter case, if the milk has been held at a low temperature, another factor is to be considered. When reproduction proceeds very slowly the newly formed cells are physiologically more mature and do not exhibit the marked sensitiveness of cells newly formed at optimum temperature.

In studying the abnormal gassy fermentations in Swiss cheese, Albus [2] has found that the physiological characteristics of the cells of the responsible organism, at the time of the manufacture of the cheese, are of great importance. An aerobic gas-former belonging to the aerogenes group of bacteria was used to produce an abnormal gassy fermentation. He found that if the cells were of such maturity when introduced into the milk that they were growing rapidly before the cooking temperature was reached, the heat caused a rapid diminution in the number of living cells and the cheese was less apt to become gassy. He later demonstrated [3] that anaerobic spore-formers, responsible for gassy fermentations in Swiss cheese, were equally susceptible under the same conditions. Incubation of the milk for a period of from 30 minutes to one hour brought the organisms into an active stage of reproduction by the time the cooking temperature was reached, and resulted in greatly diminishing the gassy fermentation or checked it entirely. He concludes that the physiological condition of the bacterial cells, at the time the cheese is made, is an important determining factor in the abnormal gassy fermentations in Swiss cheese. The efficiency of the starter employed to combat abnormal fer-

mentations was found to be dependent upon the stage of maturity to which the cells of the bulgaricus organism were allowed to develop.

Chemical changes. That during multiplication and with increasing age a culture may show considerable changes in the chemical composition of the bacterial cells has been demonstrated by Hirsch[185] in experiments with the diphtheria bacillus. Variations in the hourly increase of bacteria were shown to be tied up with changes in the chemical composition of the bacteria. These changes were not constant in one direction but were fluctuating. Buchanan and Fulmer[52] (Vol. I, p. 77) review work which shows that the chemical composition of bacteria varies with the age of the organisms and with the medium in which they are grown. Habs and Blau,[146] however, report that the composition of the bacterial cell does not depend on the nitrogen content of the medium.

There is a common belief that changes in titratable acidity and hydrogen-ion concentration follow the growth curve. To what extent this is true is indicated in the work of Rahn,[327] and of Cohen and Clark.[70] Rahn grew *Streptococcus lactis* in milk but could detect no change in titratable acidity in the early stages of growth. He offers a method for calculating the fermenting capacity of the average single cell and concludes that there is not a sufficient number of cells in the early stages of growth to produce a titratable amount of acid. Cohen and Clark grew *Bacterium coli* in glucose broth. The first change in pH did not occur until the culture was well along in the logarithmic phase of the growth curve. The possible influence of the buffer content of the medium must not be lost sight of in considering work of this kind.

Effects of various factors on growth. It is not to be supposed that the development of a cell population should proceed at a uniform rate. Thus in the growth of a bacterial cell population we have a process which at first, for a very brief period, takes place slowly, then with a greatly accelerated velocity, followed by a sharp return to a slow reproduction until the maximum population is reached. These fluctuations of growth comprise the bacterial growth curve. The curve is of the same general nature for all bacteria under conditions favorable to growth. The extent and slope of the curve is, however, sensitive to unfavorable influences. Any factor or group of factors which affect normal bacterial growth will cause a more or less irregular growth curve. Thus the growth curve not only reflects the influence of factors which affect the growth of the organisms, but also indicates the extent or severity of that influence. Certain factors may be considered to have a general effect in that their influence is shown throughout the entire length of the growth curve. The effect of other factors seems to be confined to certain portions or phases of the growth curve. These factors will be taken up in a discussion of the various phases of bacterial growth.

The most important single factor affecting the growth of bacteria in a favorable nutrient medium is temperature. Any deviation from that temperature which is optimum for the growth of the organism or organisms in question affects the growth and consequently alters the growth curve.

Barber,[19] working from single cells of *Bacterium coli,* found that as the temperature increased from 10° to 37° there was an increase in the rate of multiplication. From 37° to 45° the rate was not much changed. However, above 45° the rate of multiplication fell rapidly and reproduction ceased at 49°. Tanner and Wallace[418] grew thermophilic bacteria at 20°, 37°, and 55°. Growth at 55° was exceedingly rapid and they noted that at this temperature the cultures were short lived. At 37° they found the growth curve to be much extended. At 20° the maximum population obtained was very low, but the cultures were viable at the end of six weeks. Sherman and Cameron[386] in work with *E. coli* found that abrupt changes in temperature of incubation of a growing culture were lethal to young cells in the culture and that a drop from 45° to 10° killed more organisms than a rise from 10° to 45°. Buchanan[207] (p. 54) and Buchanan and Fulmer[52] (Vol. V, p. 33) discuss in more detail the relation of temperature to the growth curves of bacteria.

The influence of temperature on the growth of bacteria is recognized in the manufacture and curing of cheese. The process of cheese manufacture, aside from the actual separation of the curd from the milk, is a fostering of the desired bacterial flora. This is accomplished by preparing a suitable medium and using such temperatures as will favor the development of the desired flora. In cooked-curd cheeses the comparatively high temperatures, which are maintained in these cheeses for many hours, favor the growth of the high temperature lactobacilli and streptococci responsible for the initial fermentations. At the same time, the growth of other organisms, notably those that produce undesirable fermentations, is checked, and in some cases large numbers are destroyed. The physiological maturity of the starter organisms will determine to a great extent the ability of these organisms to survive the cooking temperatures and to begin growth and fermentation at the proper time in the cheese.

From the standpoint of its effect on bacterial growth, and consequently upon bacterial growth curves, the influence of the nutrient level of the medium rivals that of the temperature. The elementary nutritive requirements of the cells are diverse. The absence of one or more nutritive element or compound must necessarily affect the normal activity of the cell. The difference in morphology of bacterial cells grown in media of high and low concentrations of nutrient are well known. Penfold and Morris[315] studied the effect of concentration of food supply on the generation time of bacteria. They found that with increased concentration of nutrient the generation time was shortened. In a medium of a low nutrient level the maximum cell population is lowered accordingly.

It has often been observed that starters prepared in whey differ from those prepared in milk, not only with respect to the titratable acidity attained but also in their activity when used in the manufacture of cheese. The maximum population attained in a starter made from whey is considerably less than that in a starter made from milk. This difference is

but the natural result of growth in a medium of lower nutrient concentration.

Milk and its products are generally considered to be adequate as nutrient media for the growth of many types of bacteria, but as has been pointed out above (p. 340) different samples of milk vary in their suitability as culture media, e.g., for preparation of starters. A number of workers have added accessory food substances to milk to improve it as a culture medium for growing starter organisms. Among the substances added were yeast extract,[304, 229, 299, 135] casein peptone,[135] extract of spinach,[229] and hydrolysate of milk.[229] A high fat milk has been found superior to a low fat milk,[229] and mastitis [460, 473] and colostrum [460] milk were inferior to normal milk. Previous growth of other organisms may favor [77, 319, 265] or inhibit [470, 471] the growth of lactic starters.

The influence of hydrogen-ion concentration of the substrate on bacterial growth has been studied by many workers, who have shown that rate and amount of growth, morphology of the cells and amount and kind of products formed vary with variations in hydrogen-ion concentration. This will be discussed in more detail in the following chapter.

The influence of bacterial symbiosis on bacterial growth is generally recognized. It is often difficult to determine whether or not the favorable action of one organism on another is due to simultaneous growth or growth of one organism ahead of the other. Many workers [264, 265, 77, 319] have reported that *B. subtilis* favors the growth of *S. lactis*. *L. bulgaricus* and *S. lactis* were found mutually stimulative in yoghurt.[442] *Oidium lactis* and other molds favor the growth of *S. lactis* according to Barthel,[25] but Landau [244] claims that *Oidium lactis* has practically no influence on *S. lactis*. Motile acetic acid bacteria in whey extract of calves' stomachs are said by Ritter [340] to aid the growth of *Bact. casei* ε and thus make a better starter for Swiss cheese. Sherman and Shaw [379] found that the activity of *Bacterium acidi-propionici* d, the organism partially responsible for the "eyes" and the characteristic flavor of Swiss cheese, was greatly increased when grown in association with *S. lactis, L. casei* and two unidentified non-lactose fermenting bacteria. *L. casei,* it will be noted, is often used as a starter for Swiss cheese. *Lactobacillus bulgaricus* in milk in association with a mycoderm had its growth accelerated, produced a higher titratable acidity than when grown alone and persisted for a longer time in the milk culture.[350]

Examples of metabiosis in dairy products are numerous. The ripening of most kinds of cheese is due to the action of a succession of different kinds of organisms. A sample of raw milk held at room temperature undergoes a series of changes due to a succession of organisms.

Antibiosis is a phenomenon familiar to the dairy industry and only a few examples can be given here. The starter bacteria are active in suppressing abnormal fermentations in dairy products, such as gassy fermentations in cheese. Certain bacteria are able to inhibit the starter organisms, but usually must start to grow ahead of the starter bacteria. *E. coli* has been reported inhibitive to *S. lactis* [470, 77] and one strain of *S. lactis*

has been found to inhibit other strains.[471, 472] Rogers[353] has shown that *S. lactis* has an inhibiting effect on *L. bulgaricus*.

The lag phase. The lag phase of a bacterial growth curve is that period through which a newly inoculated mature culture may pass before reproduction begins. Lag was first recognized by Müller.[278] Several views have been advanced to explain the nature of lag. An early view considered lag to be a fault of the culture medium. Robertson regards lag "as an inevitable consequence of isolation." Barber,[19] Penfold,[316] and Chesney[58] have definitely shown that lag is due to a change in the cells themselves. They demonstrated that transplants from rapidly growing cultures give no lag. Chesney further showed that lag occurs when transplants are made immediately after the period of logarithmic growth is past. Chesney's findings led him to the view that lag was an expression of injury received by the cell from its previous environment. Sherman and Albus[383] found that cells of *Bacterium coli* one and one-half hours old were sensitive to the action of 5 per cent NaCl although no measurable increase in numbers had taken place. Thus the mature cells assumed the characteristics of young cells before reproduction began. This fact, they believe, "justifies the view that during the lag period the old cells undergo a biologic rejuvenescence which fits them for reproduction." Such a view receives support from the work of Henrici[182] who noted that the bacterial cells undergo significant morphological changes before reproduction begins.

The length of the lag period is constant for the same organism under identical conditions. The period can be extended by any factor or factors which retard growth. Cohen and Clark[70] found that the lag period was longer for the same organism grown in an alkaline medium and Graham-Smith[140] found that the addition of acid or alkali to the medium tended to retard growth during the earlier phases but not during later phases. The size of the inoculum and the age of the mother culture have a great influence on the length of the lag period. An abbreviation of the lag period of certain bacteria has been reported.[10, 375] Curran[81] found that the presence of glucose and increases in the concentration of peptone solutions shortened the lag period of *E. coli* and that a large series of compounds added in small quantities tended to prolong the lag phase. Walker[464] claims that lag is due chiefly to the time for a culture to build up carbon dioxide. Rahn and Ferguson[328] think that mitogenetic rays from the organisms may shorten the division time, hence the more organisms in an inoculum the greater the radiation and the shorter the lag period would be. Aeration[480] has been shown to prolong the lag of *E. coli* for a short time in a rich medium and for many hours in a poor medium. Salts, like aeration, apparently hindered the cells during the lag period and stimulated them during the logarithmic phase.

Walker and Winslow[465] working with *E. coli* found an enormous increase in metabolic activity per cell, especially in ammonia production, toward the end of the initial lag period. This increase was over the rate during the period of stable population and was greater than during the

phase of logarithmic increase. Later work [466] indicated that the increase in activity per cell could be accounted for partly but not entirely by the increase in the size of the cells late in the lag period and the decrease in metabolic activity per cell during the logarithmic growth phase could be partially explained by a gradual decrease in cell size.

The logarithmic phase. The logarithmic phase of the bacterial growth curve is that part in which the rate of increase per organism remains constant, that is, the minimum average generation time is maintained throughout the period.[52] It begins at the end of the positive growth acceleration phase and continues to the time when the bacteria continue to increase in numbers, but less rapidly than during the logarithmic growth phase. At the end of the phase of negative growth acceleration the cell population has approached its maximum. The autocatalytic character of this portion of the growth curve has led Robertson [343] to believe that the cells are mutually accelerated by some accelerative material discharged by them into the surrounding medium. This mutual acceleration he has termed the "allelocatalytic effect." Neither Sherman and Albus [382] nor Curran [81] were able to demonstrate an allelocatalytic effect when *Bact. coli* was transplanted from rapidly growing cultures.

It will be seen from works previously cited that during the logarithmic period the cells possess certain definite characteristics. Morphologically the cells are much enlarged. The diphtheroids have been found to be an exception in this respect and possibly other exceptions can be found. Physiologically the cells are more sensitive than mature cells, and when transplanted into fresh medium of the same nature and temperature, they exhibit no lag and a slightly higher maximum cell population is attained.

As has been pointed out above, it has been shown by Walker and Winslow [465] that there is a greater metabolic activity of *E. coli* per cell during the logarithmic phase than during the period of stable population as shown by a 36-fold increase in formation of carbon dioxide and a 20-fold increase in formation of ammonia nitrogen.

Cohen and Clark [70] studied the growth of certain bacteria in media of different hydrogen-ion concentrations. According to their observations there is a broad zone of pH in which rates of growth are quite uniform but on the borders of these zones a slight shift in pH markedly affects the reproduction. The acid border shifts with the nature of the acid. It is of particular interest to note from their work that the growth of *L. bulgaricus* was checked at pH 4.5 although the strain employed was capable of bringing its culture medium to a pH of 3.9. *Bact. coli* was found to be capable of bringing its medium to a pH of 4.5. These facts have a possible significance in the manufacture of the cheeses in which a bulgaricus starter is used. Hall and Fraser [149] studied the effect of dilute acids on bacterial growth in optimum hydrogen-ion concentration, and found the effect to be confined to the logarithmic phase of the growth curve.

The resting phase. The senescent or resting phase of the growth curve begins with the cessation of active reproduction and continues until

the decrease in cell population begins. During this period the cells acquire the characteristics of maturity and the maximum cell population is attained. Morphologically the cells revert to the size of resting cells. Physiologically they gradually acquire the characteristics of mature cells and, as shown by Chesney,[58] the length of "lag" increases to the normal as the period progresses. This illustrates the importance of the relationship between the age of a culture and the presence of lag, a point that might well be considered in the use of starters.

The nature and origin of those influences which limit the bacterial cell population inhabiting a limited volume of nutrient medium affords one of the most perplexing problems. The most obvious supposition was that the number of cells increased until the available nutriment for each individual was insufficient to furnish the excess food material necessary for the manufacture of new protoplasm. However, it has been shown by a number of investigators that the exhaustion of available food material is not alone a determining factor in cell population. Likewise the cell proximity, or crowding, has been shown not to be, in itself, responsible, although Curran [81] reported that density of bacterial population appears to be a limiting factor in the growth of bacteria under certain conditions.

In any given medium the same strain will reach a definite maximum population. According to the work of Bail,[14] the maximum cell population attained by one type of organism in mixed culture, is dependent upon the number of cells of the other type inhabiting the same medium. Thus a starter may check the development of a maximum population of an undesirable organism, not only with the acid it produces but also by virtue of the number of cells, i.e. the earlier attainment of a maximum population. This can be accomplished by a "mass" inoculation with a starter which is of an age to assure early reproduction.

The accumulation of toxic products within the medium has frequently been considered as a factor limiting bacterial growth. Of particular interest here is the work of McLeod, Gordon and Pyrah [261] who, among others, have demonstrated the formation of peroxide by certain bacteria. They found that *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* produce peroxide and they suggest that all bacteria that produce lactic acid primarily, produce peroxide. They found the concentration of peroxide in the cultures with which they worked to be sufficient to inhibit growth of the organism.

Rogers and Whittier [351, 352] have recently investigated the effect of various factors on the maximum cell concentration of *Streptococcus lactis*. They were able to increase the cell concentration by maintaining a constant pH in the medium, showing that pH may be one of the limiting factors. They found a direct relation between the number of bacteria, the cessation of fermentation, and the final concentration of undissociated lactic acid when the organism was grown in milk or whey. They point out, however, that apparently no such relation exists when the organism is grown in an artificial medium. These authors further found that aeration increased the cell population and that when nitrogen was used instead of air a still

higher level of cell concentration was obtained. The reduction potential was found to have no determining influence.

Growth retarding substances from old bacterial cultures have been reported by a number of workers. These substances are usually either volatile or thermolabile, for heating the medium destroys their effect. Robertson [348] assumed the progressive accumulation of autocatalyst in the medium to be the limiting factor that inhibits reproduction. Rogers and Whittier [352] found that a substance diffusible through a collodion membrane limits the growth of *S. lactis,* but Frazier and Boyer [124] reported that the filtrate from an old culture of *S. lactis* greatly stimulated the growth of lactic acid bacteria.

The death phase. The death phase has its inception in the first permanent decrease in the cell population and terminates when no living cell remains in the culture. Biologically viewed, this phase of the bacterial growth curve is of considerable interest, but does not warrant detailed consideration in this work. The slope of the curve is rather gradual, depending upon such conditions as the nature of the organism, temperature, reaction and other factors. It has been observed that during this period many of the cells of the community assume unusual morphological characteristics. Transplants from this phase exhibit a normal lag and a normal growth. Reported observations to the contrary may have been due to a lack of proper consideration of the number of living cells in the inoculum.

Figure 36, from the work of Chesney,[58] is an example of a typical growth curve. It illustrates the growth of a culture of *B. coli* in bouillon at 37° and of subcultures taken from the parent culture at various stages of growth. It is to be especially noted that subcultures taken during the logarithmic growth phase exhibited no lag, but continued to grow at the same rate as the parent culture; also that subcultures taken after the parent culture had passed its period of rapid growth exhibited a lag which became longer as the rate of multiplication in the parent culture decreased and the number of mature cells increased.

Recognition of the various phases of bacterial growth and of the characteristics of the cells at the various stages finds ready application in the preparation and use of starters, in controlling the various processes of manufacture of dairy products, in pasteurization and sterilization, and in the determining of bacteriological technic.

Spore Formation and Germination

Bacterial spores as well as the vegetative forms are constantly present in milk and many dairy products. The origin of most if not all spore-forming bacteria is the soil. Under the usual conditions of milk production and handling, spores are not formed in milk but enter largely through the agency of dust, feed, and utensils. Market milk usually contains the spores of the subtilis-mesentericus group. Obligate anaerobic spores are rarely absent from milk, though their numbers are usually small.

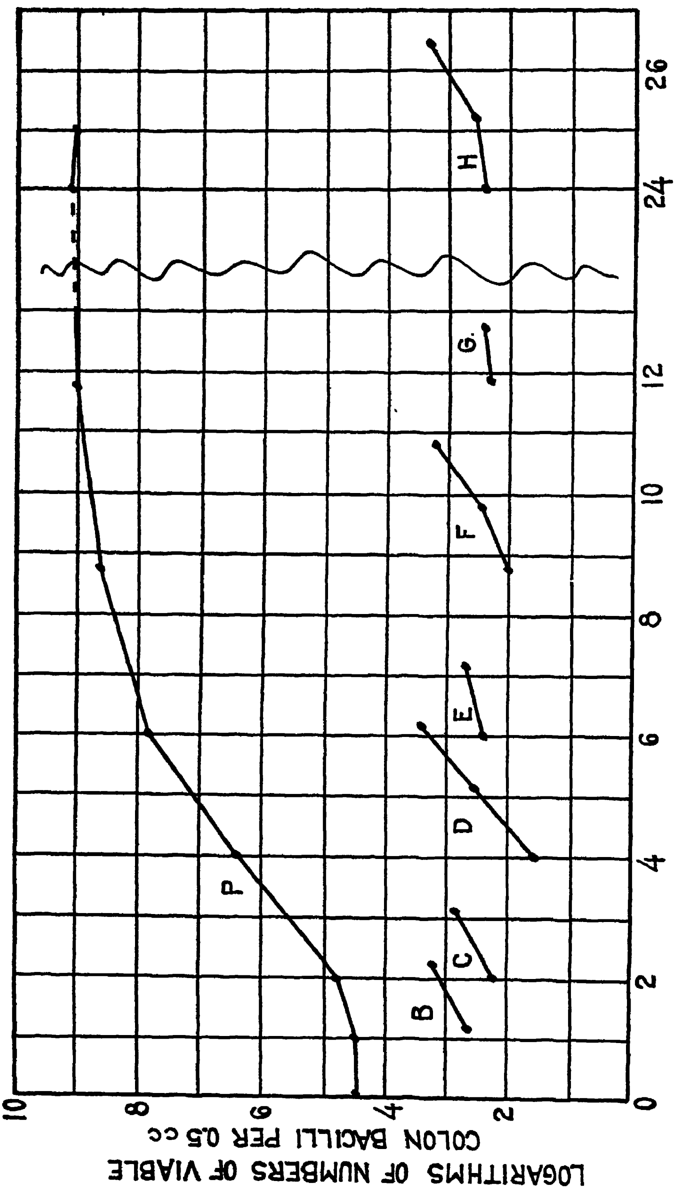

FIG. 36.—Growth of parent culture and subcultures of *B. coli* in bouillon at 37° C. P indicates parent culture. B to H indicate subcultures. From Chesney.[58] (Courtesy Journal of Experimental Medicine.)

(Hussong and Hammer.[202]) *Cl. welchii, Cl. sporogenes,* and *Cl. butyricum* are the most frequently encountered. Experimental evidence indicates that none of these germinate in milk handled under market milk conditions. Spores of both aerobic and anaerobic thermophilic bacteria are found in milk and frequently cause trouble in pasteurization. Milk to be used in the manufacture of cheese or evaporated milk is sometimes held over some period of time. If a low temperature is not maintained throughout this period both aerobic and anaerobic spores may germinate, producing sweet curdling, proteolysis and other kinds of spoilage. All spoilage losses which occur in the manufacture of unsweetened condensed milk, with the exception of those which are the result of defective cans, are due to the survival and subsequent development of heat-resistant spores. Both mesophilic and thermophilic aerobes and anaerobes have been frequently isolated from such outbreaks. In the manufacture of some types of cheese anaerobic spores are frequently the cause of serious defects. *Cl. welchii, Cl. butyricum,* and *Cl. putrificum* are those usually encountered. Spores of these bacteria have been shown to be especially numerous in milk from cows fed on silage, meal, earth nuts, etc. (Burtscher.[55])

Characteristics of spores. The spores characteristic of certain bacteria may be regarded as an extreme example of resistance in mature cells. In their vegetative form these organisms (the genera *Bacillus* and *Clostridium*) are no more resistant to heat than other bacteria, but once the spores are formed they can survive boiling temperatures for considerable periods. A temperature of 115° to 120° is usually necessary for prompt sterilization. Occasionally one encounters strains so refractory that even this degree of heat can be borne for several minutes. Spores also resist chemical treatment by disinfectants and dyes. Treated with ordinary stains the spore remains colorless; but if the refractile membrane around it be injured by flaming or by acids, coloring matter may be driven in, especially by heat, and is then not readily washed out. These peculiarities depend on several factors. It is usually believed that the substances within the spore are more concentrated than in the vegetative cell and in somewhat different proportions. Virtanen and Pulki [458] found that the spores and vegetative forms of *B. mycoides,* however, did not differ in percentage of water, ether soluble material, hydrolyzable carbohydrate, nitrogen, or ash. There is some evidence that the nature of the protein has been changed, as judged by the most delicate tests. Chimera [59] and Mellon and Anderson [270] found that the spores of *Bacillus subtilis* are not agglutinated by sera which agglutinate the vegetative cells and vice versa. The resistance to heat may be due in part at least to increased concentration, if a difference does exist, as it is well known that proteins are the more easily coagulated by heat the more dispersed. Virtanen and Pulki [458] believe that differences in enzymic activity may account for thermostability of spores. These workers found that the most vigorous catalase and glucolytic enzymes were absent from the spores of *B. mycoides.* Spores can endure drying and absence of food for years. Silk threads dipped in anthrax cultures forty years ago in Koch's laboratory still contain live

spores capable of germinating if dropped into broth. Obviously an organism in which metabolism is at a low ebb is less reactive to the influence of changes in its environment. Spores, as such, are relatively sluggish in their vital activity but in no sense inactive. Ruehle [357] examined spores from 12 aerobes and found that all showed catalase and in some cases evidences of gelatinase and possibly a little caseinase and lipase. Cook,[78] working with the spores of *B. subtilis,* demonstrated the presence of a marked glucose oxidase and a proteolytic enzyme more pronounced in spores than in vegetative forms. Tarr [422] found that the spores of aerobic bacilli dehydrogenate certain mono- and di-saccharides anaerobically.

In the strict sense of the word bacterial spores are not reproductive. Each rod produces but one spore (except in a very few exceptional cases) and the spore germinates into a single unicellular organism. However, like the spores of molds and the seeds of the higher plants, they can be carried about by various agencies and, when dropped in a favorable environment, they start new colonies thus spreading the species abroad. Spores as such are relatively inactive and innocuous but this ability to withstand privation and destructive agencies and subsequently to germinate makes them an important problem in medical and industrial bacteriology.

Conditions influencing sporulation. The factors governing sporulation and germination are by no means completely understood. What is known has made possible various useful technical expedients, and further research may well introduce others of great industrial importance.

Early observation indicated that vegetative growth and sporulation do not go on at the same time—evidently the conditions favoring the one inhibit the other. From this observation and the knowledge of the resistance of spores, the plausible assumption was made that sporulation is a response to unfavorable conditions, particularly lack of food. That this idea is at least partially erroneous, and even if true is far from the whole truth, later became obvious. It is a fact that rapid transfers of a vigorously growing culture, before the nutriment is exhausted or sporulation has set in, will prevent sporulation and will even, in some cases, favor the development of temporarily asporogenous strains. On the other hand, certain slightly unfavorable conditions insufficient to interfere seriously with multiplication, and with no effect at all upon the spore once it has been formed, inhibit sporulation. For example, sporulation of *Bacillus anthracis* is prevented by growing it at 42° or in the presence of extremely small quantities of phenol or bichromate. A process so easily prevented by mildly injurious agents can hardly be regarded as a response to unfavorable conditions. Moreover, it has been shown that maximum sporulation, both as to gross numbers of spores produced, and intensity (ratio of spores to cells) is greater on media which are amply adequate to support vigorous vegetative growth. In such a culture left to mature naturally, sporulation may take place before all the nourishment is used up, for filtrates from such cultures often support growth when reinoculated. In an inadequate medium where growth is scarce sporulation may set in

sooner than in a rich one, but not all the cells will sporulate, and not all the spores will be morphologically typical. What, then, is the impulse which initiates sporulation? Can it be simply a lack of food? Williams [476] found that the percentage of spores formed by *B. subtilis* when grown in 5 per cent peptone water was much lower than when the same organism was cultivated in 0.5 per cent peptone water. Tarr [421] confirmed this principle and concluded that the major factor controlling the formation of spores by aerobic bacilli on solid media is the amount of available nutrient material. Thus, in a medium containing relatively large amounts of available nutrients, few or no spores were formed, but when sufficiently diluted with inorganic salt solution the percentage of spores greatly increased and frequently attained 99 per cent total if the dilution was sufficiently high. That this is not always true is shown by the work of Brunstetter and Magoon [49] in which spore formation by *B. fusiformis* increased as the concentration of food in the medium increased. These investigators believe that it is illogical to consider the amount of available food in the medium as a factor in promoting sporulation without also considering the concomitant factor of available oxygen. Under ordinary cultural conditions an increased concentration of nutrients increases the amount of growth which in turn results in a lowering of the oxygen tension until finally a point is reached where sporulation becomes impossible. Some investigators have maintained that the accumulation of a sufficient quantity of some end product of metabolism produces the stimulus. No such product has ever been isolated from a culture, however. The work of Williams [476] and of Tarr [421] indicates that depletion of nutrient materials is more important in promoting the formation of spores than is the accumulation of metabolites.

The influence of salts upon sporogenesis has been studied by Fabian and Bryan [114] and others. Univalent cations were found to stimulate aerobic spore formation, but di-, tri- and quadri-valent cations had no stimulating action on sporogenesis. The fact that spore formation was most abundant at the point of maximum viability suggests that neither a deficiency of nutrient materials nor accumulation of metabolic products is the primary cause of spore formation.

There is considerable evidence that sporulation sets in when some factor or group of factors suddenly checks the vegetative growth of a fairly mature, well-nourished culture. For example, cells transferred from such a culture to distilled water, or treated gently with fumes of certain fat solvents, can be made to sporulate much sooner than if left in the culture. This is not true, however, if the cells are removed during the first hours of rapid growth; a certain maturity seems to be necessary. There are other factors necessary to sporulation, though they are not of themselves sufficient to incite it. A hydrogen-ion concentration within suitable limits is one of these. Anaerobes are particularly sensitive in this regard, and, while an addition of sugar greatly encourages their growth, usually, however, sporulation in pure culture is prevented or reduced in the presence of the acid produced from a fermentable carbohydrate. But, as

shown by Simonds,[388] in mixed flora sporulation may occur even in the presence of free acid or fermentable carbohydrate. Thus symbiotic influences are of vital significance in spore formation. Daranyé [89] believes that the most important favorable factor for spore development is a dehydration of the colloids of the cells. With artificial dehydration produced by alcohol vapor or calcium chloride this investigator was able to cause young developing bacilli to form spores rapidly. Without such treatment spores were formed only after aging which it is believed reduces the water content of the cells. The range of temperature favorable to sporulation is narrower than that which permits good multiplication. The two factors (hydrogen-ion concentration and temperature) may be interrelated, as shown by Itano and Neil.[206] Torrey, Kahn, and Salinger [484] found that *Cl. welchii* growing in a sugar-free well-buffered fluid medium sporulated from pH 6.8 to the alkaline limits of growth and sporulation was entirely checked in reactions more acid than pH 6.6. Heat in the form of pasteurization has been found to stimulate spore formation in certain anaerobes. (Glothe and Cunningham.[180]) An abundant oxygen supply is absolutely essential for the sporulation of aerobes. The methods of preparing spores by dipping threads or cardboard or gypsum blocks in broth cultures combine increased aeration with a suddenly decreased food supply and consequent check of multiplication. The sporulation of anaerobes can sometimes be hastened by the access of air, possibly due to the check of vegetation by the oxygen, possibly to some specific effect it may have on spore formation. It is impossible to state definitely at the present time that spore formation is brought about by any one physical or chemical effect. Neither the amount nor range of experimental data upon this subject is sufficient to explain the exact mechanism of spore formation. An excellent review of the literature dealing with factors which influence spore formation is given by Cook.[74]

Conditions influencing resistance of spores to heat. Spores of different strains of the same species show variations in resistance to heat and to germicides; and individuals in the same culture are not all of uniform resistance. Such individuals may cause isolated instances of spoilage in supposedly sterilized products. The influence of various factors upon the heat resistance of spores has received considerable attention in recent years. Dickson et al.[98] made the important observation that mineral oil layered over broth extends the thermal death time of spores heated in this medium. Williams,[475] Sommer,[392] and others have emphasized the importance of the nature of the nutrient material in the production of heat-resistant spores. The former investigator found that spores of diminished resistance were formed in all digest media with the exception of casein digest. Resistant spores were formed in isoelectric gelatin and vegetable infusions. Low salt media seemed to contribute to high resistance in spores. Cultivation in the presence of a suitable concentration of either phosphate or magnesium resulted in the formation of spores more resistant than normal spores.

The heat resistance of a spore suspension increases with density but

beyond one billion per cc. this is constant according to Sommer [392] who worked with spores of *Cl. botulinum.* Magoon [262] observed that spores attained the highest resistance to heat when formed under conditions of moderate temperature and humidity. That other factors in environment contribute to the development of resistance in spores is shown by the recent work of Curran [85] who found that the prolonged action of growth-products reduced materially the heat-resistance of spores. Natural conditions of habitat were particularly conducive to resistance in the spores. Spores formed and held for several months in soil and oats were considerably more resistant to heat than spores similarly formed and held the same period of time on artificial media. Aging probably increases the heat-resistance of spores of most species although variable results have been reported. The reaction of the medium in which spores are heated profoundly influences their heat-resistance. Maximum resistance is usually exhibited near the neutral point. Magoon,[263] and Williams [475] have shown that the resistance of spores of *B. mycoides* and *B. subtilis* to heat can be increased by a process of selection of the resistant cells. Sommer [392] was unable to accomplish this with the spores of *Cl. botulinum.* An excellent account of the effect of disinfectants on bacterial spores is given by Chick.[269]

Conditions influencing germination of spores. Spores rarely germinate if left undisturbed where they were formed, and, when they do, it is only after considerable interval in which some chemical·readjustment may have taken place in the media. Such a change has been accomplished by heating.[94] Transfer to fresh suitable media at a favorable temperature and pH usually induces prompt germination. Even under such circumstances, there may be considerable variation in the time required, which may be greatly prolonged in the case of certain individual spores. This delay of germination or dormancy may be the cause of serious discrepancies in experimental work, and is a serious complication in fractional sterilization. The cause of dormancy in spores is unknown. It has been suggested that spores like seeds require a period of maturation after being set free from the mother sporangia but the work of McCoy and Hastings [259] would seem to disprove this; for anaerobic spores with dormancy of only 19 days were derived from agar colonies 96 hours old, while spores with the extreme dormancy of 222 days came from a stock culture in corn-mash 1 year old. Earlier workers attributed dormancy to heat inhibition, more recently Burke,[54] and McCoy and Hastings [259] demonstrated that in many species there is a factor of normal dormancy evident in unheated spores. Probably the most plausible theory would explain dormancy on the basis of relative permeability of the spore-wall. Dormant spores are believed to possess a relatively thick impervious cell wall which would not only make them less responsive to growth-stimulating substances but also more resistant to heat and there is considerable evidence to indicate that the more dormant spores are the most heat-resistant. Rogers and Curran [354] have recently demonstrated the existence of a substance, apparently widely distributed in nature, which

exerts a profound stimulating action upon the germination of certain spores. Extremely small quantities of concentrated aqueous extracts of liver, potatoes, yeast, mold mycelia or other substances were found to increase the spores which will germinate in milk in a short period several hundred fold. The maximum effect was obtained only when the milk, extract, and spores were heated to 100° for 10 minutes prior to incubation. The extracts were concentrated by precipitation with lead acetate after the method described by Tatum, Peterson, and Fred.[424] The importance of heat in rendering the extract effective suggests that the latter may function by altering the permeability of the spore envelope. Treatment by flowing steam on each of three successive days is a time-honored method for substances which can not stand temperatures above boiling. Vegetative cells only are killed during the heating; the success of the maneuver depends upon germination of the surviving spores in the intervals between treatments. Spoilage due to spore-bearers in milk products can sometimes be avoided by actually incubating the milk before it is heated. This is, of course, advisable only when the danger from spore-bearers appears more of a menace than the acidity or other changes which bacterial multiplication will induce in the milk during incubation. Another point of some importance is the empirical observation that, with some species at least, "shocking" spores—that is, a brief, severe, but sub-lethal heat treatment—seems to cause them to multiply with increased vigor when germination occurs. This has become a routine procedure in preparing inocula for some fermentation processes. On this basis one can easily see why an incomplete steam sterilization of machinery or containers may do more harm than good, when the organism chiefly to be feared is a spore-bearer. Its vegetative vigor may be enhanced by the very means that remove the competition offered by the presence and multiplication of less harmful non-sporulating organisms. It should not be assumed that temperature is the most important factor governing germination. The same factors that govern sporulation enter in, though their relative importance is different, and their optima are at different levels.

Access of oxygen will inhibit germination of obligate anaerobes; Fildes [117] has shown that the germination of spores of *Cl. tetani* depends mainly upon the reducing intensity of the medium, the greater the reducing intensity the shorter the lag and vice versa. Itano [206] and others have found that differences in pH are less important in germination than in sporulation. In general, the oxygen and pH minima for vegetative growth and germination are somewhat lower than the oxygen and pH minima for spore formation (Wund [487]).

The maximum and minimum temperature for germination varies, of course, with the species. Blau,[40] and Itano and Neil,[206] and others studied the influence of temperature in relation to spore germination. The latter investigators observed that the spores of *B. subtilis* failed to germinate at 50°. At 25° and 37° germination occurred only when the hydrogen-ion

concentration of the broth was kept between pH 5 and pH 10, but not at higher or lower pH values.

Free moisture is essential to the germination of spores. Milk powder and other dried foods keep indefinitely so far as bacterial spoilage is concerned, in spite of the large numbers of spores they contain.

Christian [60] isolated an interesting spore-forming organism from commercially sterilized milk. It was found that, if the spores were inoculated into milk and the spores heated, germination invariably occurred. If, to a small culture of heated spores having the power to germinate, a small quantity of living culture of the vegetative form was added a number of the spores lost their power to germinate. A stable vegetative form apparently produces a heat-labile growth factor inhibitory to the germination of the spores.

An important factor in the germination of spores is osmotic pressure. Curran [82] confirmed Holzmüller's observation [190] that the osmotic tension necessary for optimum germination is lower than that required for maximum vegetative development but found no evidence of a purely osmotic limitation in low pressure nutrient solutions. He concluded that a deficiency in food is probably the limiting factor in most low pressure solutions. When the minimum nutritional requirements for optimum germination were exceeded, the addition of even minute amounts of food substances retarded the rate of germination due to the unfavorable osmotic pressure created by the added nutrients. Under these conditions a certain degree of dilution accelerated the rate of germination but, when the nutrient concentration was below the minimum threshold, the addition of small quantities of nutrients increased the rate of germination.

Eijkman,[110] and Curran [82] found a definite maximum osmotic pressure for germination. Unless compounds with a definite chemical toxicity were used the concentration necessary to inhibit germination differed with the substance used but usually had similar osmotic pressure as measured by depression of the freezing point. Surface tension is not a significant factor in the germination of bacterial spores as shown by the work of Curran,[83] who found that the germination of spores of *B. mycoides* were only slightly affected by wide variations in surface tension.

Products of Bacterial Metabolism

Bacteria, as parasites on living plants and animals and in their manifold activities in soil, in foods, and in the fermentation of vegetable material, form an infinite variety of by-products. Our present interest, however, is especially with the results of bacterial activity which affect the flavor or the physical condition of milk and its products. The distinctive flavors, both desirable and undesirable, in dairy products are almost always the result of chemical changes brought about by bacteria and other microorganisms.

Classification. The more important groups of the flavor-producing chemical compounds are:

1. Hydrocarbons. These are produced by the higher plants and include such highly flavored substances as wintergreen, oil of nutmeg, pennyroyal, etc. They are not formed by bacterial action and are not of importance in dairy products.

2. Alcohols. Various alcohols are produced in the fermentation of carbohydrates by a great variety of bacteria and especially by yeasts. They are important as flavor-producing substances in their combinations with the acids to form esters.

3. Aldehydes and ketones. These are produced by bacteria in the decomposition of carbohydrates. Some of them have very penetrating flavors and odors and are probably the immediate cause of many of the off flavors of dairy products.

4. Acids. Acids are produced by bacteria in great variety in the fermentations of sugars and other carbohydrates, in the splitting of glycerides, and in the decomposition of proteins. Lactic, acetic, butyric, formic, propionic, and succinic are those most frequently produced. Many of these acids have a distinctive flavor of their own and in their combinations with alcohols are of great importance in producing flavors in cheese and in other dairy products.

5. Esters. Many of the esters have pronounced and characteristic odors and flavors. Among the best known are ethyl butyrate and amyl acetate, both of which have characteristic fruity odors. These compounds are formed in a great variety of bacterial fermentations, and account for many of the flavors, both desirable and undesirable, in dairy products.

6. Nitrogen and sulfur compounds. These include: (a) The amino and imino derivatives, many of which are highly flavored. In this class of compounds are indol and skatol which are associated with the unpleasant odors of putrefaction. (b) Hydrogen sulfide and mercaptans which are characteristic of deep decompositions of nitrogenous bodies. (c) Ammonia which has a well-known penetrating odor. It is produced in the ripening of cheese, sometimes in such amounts that its flavor is perceptible.

Lactic acid production. The ability to ferment sugars with lactic acid as a major product is common to many species of bacteria, but the lactic fermentation in milk and milk products is almost always the result of growth of one of a limited number of bacterial types.

In the true lactic acid fermentation as produced by *Streptococcus lactis* and certain other organisms, lactic acid is the principal end-product, and other products may be present in small amounts or only in traces. In the mixed lactic acid fermentation as produced by *S. kefir, B. coli, A. aerogenes,* the aroma-forming organisms and many other milk bacteria, numerous end-products may be formed in addition to lactic acid, including carbon dioxide, hydrogen, acetic, propionic, succinic, formic and butyric acids, acetone and ethanol. These two general types of lactic acid fermentation have been referred to by the terms homofermentative and heterofermentative, respectively.[227]

S. lactis, the typical lactic acid organism, forms mostly lactic acid with only a little acetic acid, and decolorizes litmus milk before curdling, forming a pink ring at the surface. *S. kefir,* on the other hand, does not decolorize litmus milk; it produces acid more slowly and forms a much greater proportion of acetic acid. Hucker[196] divides the lactic streptococci into two groups on the basis of the type of acid produced; the aroma group and *S. kefir* type produce *l*-lactic acid, form a relatively large percentage of volatile acid, hydrolyze lactose more rapidly than acid is produced, are stimulated by traces of yeast extract, and do not produce large amounts of acid. Organisms of the other group, which includes *S. lactis, S. pyogenes,* and similar organisms, produce *d*-lactic acid, form acid as rapidly as the lactose is hydrolyzed, usually produce large amounts of acid, and are not stimulated by yeast extract.

The first group includes the *Betacocci* as classified by Orla-Jensen [303] on the type of acid produced; Hammer's aroma producers, *S. paracitrovorus* and *S. citrovorus*, also belong in the first group. *S. cremoris* (Orla-Jensen),[303, 298] an aroma-forming organism, is important in cream ripening and in starters for butter making. The aroma bacteria produce as much as 40 per cent of the acid as volatile acid, while *S. lactis* and closely related types produce less than 10 per cent of the acid as volatile acid.

The form of lactic acid produced is usually constant for a given organism, regardless of the nutrient medium.[303] Henneberg,[179] however, reported that *Thermobacterium lactis* produced the *l*-form from certain sugars and the *d*-form from others. Other workers [208] found that *Bact. bulgaricum* produced the *l*-form in milk and inactive acid in another medium. Moreover, certain bacteria, when grown in association with other types of organisms, may produce a different form of lactic acid than when grown alone.[314, 423]

Acid normally forms the most rapidly and coagulation begins in the upper layers in whole milk; the numbers of *S. lactis* organisms are greater in the upper layers because the organisms are carried upward by the rising fat globules.[370] In skim milk, however, the activity of true lactic acid bacteria is greater in the lower layers.

The true lactic bacteria are included in two groups, the streptococci and the lactobacilli. In spontaneous souring of milk at ordinary temperatures, the streptococci usually predominate even in the early stages. As the acidity increases, other types of bacteria are suppressed and, when the fermentation is finally checked at a pH of 4.8 to 5.0, the flora consist almost entirely of the lactic streptococci, mostly of the *S. lactis* type. *S. bovis*, the typical streptococcus of the bovine intestine, and *S. pyogenes*, the cause of many septic conditions, may be present in the milk but have no significant part in the lactic fermentation. In the early stages of the souring, *S. kefir*, the gas-forming streptococcus, usually constitutes about 75 per cent of the total number of streptococci. The remaining streptococci are mostly of the *S. lactis* type. This species multiplies very rapidly and by the time the milk is curdled they make up about 90 per cent of the total number of streptococci, the remaining 10 per cent being almost exclusively of the *S. kefir* type.

If spontaneously soured milk is allowed to stand, especially at comparatively high temperatures, it undergoes a second lactic fermentation induced by members of a group of non-spore-forming bacilli of which *Lactobacillus bulgaricus* is a type species. Many of these organisms are very active acid formers, some varieties increasing the acidity of milk to over 2 per cent lactic acid. Certain strains, when grown for one month at 29°, have been found to produce 3.25 per cent acid calculated as lactic acid, and a hydrogen-ion concentration represented by pH 3.23.[366] These bacteria probably take little part in the ordinary souring of milk but they are of importance in fermented milks and in the ripening of many varieties of cheese.

The normal acid fermentations by the streptococci and lactobacilli are

soon stopped by the accumulation of by-products. The semi-solid, curdled milk then becomes a favorable medium for still another fermentation induced by molds.[360] *Oidium lactis* is the most common of these and forms a velvety layer on the surface, converting the acids to carbon dioxide and water and making the product more alkaline. Putrefactive organisms, which have remained inactive in the sour milk, now find conditions favorable for their growth, and the milk undergoes still another decomposition which is marked by the production of foul-smelling end-products.

If the true lactic acid organisms are low in numbers or absent, and members of the Escherichia-Aerobacter group predominate, a so-called gassy fermentation will take place, characterized by the production of carbon dioxide and hydrogen. Temperatures near 37° favor this type of fermentation. The products of fermentation by these bacteria include a large proportion of acetic and other volatile acids. They do not bring about what may be considered a normal souring of milk and are not classed with the true lactic bacteria. Karström[209] reported that a coli organism produced 35 per cent lactic, 10 per cent succinic, 10 per cent acetic, 2 per cent formic and traces of propionic acid, and 10 per cent ethanol, from glucose.

B. coli also produces carbon dioxide and hydrogen;[1] certain organisms, including *B. lactis aerogenes,* produce acetylmethylcarbinol and butylene glycol.[269] Formic acid is produced by bacteria which produce carbon dioxide and hydrogen from sugars, and it is probably the chief source of gaseous hydrogen in bacterial fermentations.[269] It is said to be formed along with acetaldehyde from the decomposition of methyl glyoxal hydrate. Organisms of the coli-aerogenes group produce more lactic acid in anaerobic than in aerobic fermentation and this is said to be due partly to an oxidative destruction of lactic acid under aerobic conditions.[452] Neuberg and Gorr[289] found that a lactobacillus produced 91 per cent of the acid as lactic acid from glucose; *B. coli* produced from 39 to 82 per cent, the amount being inversely proportional to the amount of oxygen present.

The proportion of volatile acids and gases produced by bacteria of the colon-aerogenes group varies with the conditions of the fermentation. The volatile acids produced are largely acetic and propionic; in some cases the volatile acids may constitute 30 to 40 per cent of the total acids. In addition to the fermentation of the lactose, the aerogenes type may ferment the citrates of milk to acetic acid, carbon dioxide, and other products. The addition of citrates to milk increases the proportion of volatile acid produced by certain organisms.[426]

Many of the micrococci are lactic acid formers but in comparison with the lactic streptococci they are inactive and play only a minor part in the early stages of the fermentation. Some of the streptococci, notably *S. kefir,* produce appreciable amounts of carbon dioxide gas, which is also liberated in small quantities by some of the lactobacilli and by the aroma-forming streptococci isolated by Hammer.[158]

In the presence of oxygen, *B. bulgaricus* forms a considerable amount of hydrogen peroxide, which inhibits the fermentation.[127]

In the souring of milk the conversion of lactose into lactic acid is not as simple as usually represented by the equation—

$$C_{12}H_{22}O_{11} + H_2O = 4C_3H_6O_3$$

in which lactic acid only is formed. According to certain workers,[293] methyl glyoxal occurs as an intermediate product; other workers,[451] however, state that neither *B. casei* ϵ nor *S. lactis* convert methyl glyoxal to lactic acid. In the decomposition of lactose to lactic acid there is an evolution of energy amounting to 24 calories per molecule of lactose.[269]

In the souring of milk by the streptococci, about one-fourth to one-third of the lactose is fermented, producing a titratable acidity of 0.8 to 0.9 per cent calculated as lactic acid. Most of this is lactic acid.

As a result of the fermentation of the lactose certain marked changes occur in the physical condition, flavor, and chemical constituents of the milk. Through the action of the lactic acid the dicalcium phosphate is progressively converted to monocalcium phosphate. The calcium caseinate which exists in a colloidal condition is converted into caseinates containing less calcium, and finally into free casein which is precipitated. In this readjustment soluble calcium lactate is formed.

When the acidity reaches a pH of about 5.3 curdling begins. The hydrogen-ion concentration remains stationary until the curdling is complete. The free casein does not combine with the acid, but as some is adsorbed the acidity of the serum does not represent the total acid formed in the fermentation.

The albumin, which exists in the original milk in a condition which permits only a part of it to pass through a porcelain filter, is changed so that it is completely filterable. The acid flavor which does not appear at any definite hydrogen-ion concentration is dependent more on the nature than on the amount of acid formed. It not infrequently happens that a culture with a high titratable acidity has a very mild flavor, while others show a sharp acid flavor with an absence of aroma.

Ropy or slimy milk. The marked improvements in methods of handling milk which have taken place in the last two or three decades have tended to control the abnormal fermentations until many of them are now rarely encountered. However, the condition known as ropy or slimy milk still continues and not infrequently occurs in epidemic proportions.[172] Ropy or slimy milk should not be confused with the stringy milk associated with pathological conditions, udder inflammation, or garget. The stringy condition of the milk under these conditions is caused by an abnormal secretion and not by direct action of bacteria.[403] Milk showing this type of stringiness may contain white masses or strings of coagulated material when drawn, while the real ropiness or sliminess is due to bacterial action in the milk and becomes apparent only after the milk has been held for some hours.[111, 172] The ropiness may be evident only as a slightly abnormal viscosity, or it may be so pronounced that it is possible

to draw the milk out in fine threads a yard long. In some cases the milk is a thick viscous mass which does not flow from the container. The ropiness may be confined to the top layers of the milk, but more commonly the entire mass is affected. The flavor is usually unchanged except as it is associated with the lactic fermentation and there is no reason to believe that ropy milk is unwholesome in any way. In fact the ropy fermentation is encouraged in the preparation of certain fermented milks and in cultures used in the manufacture of Edam cheese. Mattick [266] has reported a condition in which an apparent ropiness or thread formation in fresh milk seemed to be due to a formation of thin films of casein or albumin at the milk-air interface.

The immediate cause of the slimy or ropy condition is the formation by bacteria of gums and mucins.[50] The gums are the more common cause and are probably produced by the fermentation of the lactose to galactan or dextran. Some of the active peptonizing bacteria produce sliminess by the formation of mucins, which are combinations of proteins with a carbohydrate radical. The development of sliminess is closely associated with capsule formation. In many cases the threads formed when the milk is drawn out contain many chains of bacteria held together by large capsules. In other cases the capsule appears to be dissolved as fast as it is formed or a more or less complete solution of outer cell walls occurs,[50] and the gummy material is diffused throughout the milk. Such a condition may accompany an unusual proliferation of bacteria, or a formation of long, tangled masses of threads or chains. When the ropiness is thus produced, it may be eliminated by thorough agitation. In some cases ropy cultures show more chain formation than non-ropy ones.[164]

Buchanan and Hammer [50] state that ropiness develops more readily at room temperature than at 37°; Stark [404] found that ropy organisms proliferate at lower temperatures than most ordinary milk bacteria. It is said that physical ropiness is most marked when the acidity is low.[266] Henneberg [181] states that a culture may tend to become slimy when the organism is transferred frequently and grown at a low temperature in milk, and that certain milks, especially those which are coagulated slowly by rennin, show an unusual tendency to undergo a slimy fermentation.

The ability to produce sliminess or ropiness in milk, beer, wine, or sugar solutions is not confined to any one type of bacteria. It is evidently a variable character which is easily acquired or lost.

Among the bacteria causing ropiness are active gelatin liquefiers, including some of the hay bacillus type. More frequently, however, epidemics of ropy milk are caused by some members of the aerogenes group or the lactic streptococci. Some of these latter organisms have been observed with such frequency in ropy milk that they have been given specific names and are recognized as a common cause of this defect. Among these are *Streptococcus taette,* the essential organism in the Swedish ropy milk, and *S. hollandicus,* which is sometimes used as a starter in making Edam cheese. Both of these bacteria may be considered as varieties of the common *S. lactis.* Organisms of the Escherichia-

Aerobacter group causing ropiness may originate in feeds.[403] Among the organisms described as the cause of ropiness are: *B. viscosum,*[166] *B. lactis viscosus,*[180, 181, 266, 361] *S. lactis var hollandicus,*[160, 164] certain Corynebacteria[180] and members of the Escherichia-Aerobacter group,[403] including *E. neapolitana,*[361, 367] *A. aerogenes*[367] and *A. cloacae.*[367] Slimy milk may result in some cases from the growth of strains of ordinary milk streptococci, as *S. lactis, S. cremoris* or *S. thermophilus.*[181] Hammer[164] found that certain strains of *S. lactis* may vary from ropy to non-ropy, or normal strains may develop into strains which produce ropiness. The ropy strains were characterized by a striking increase in their air requirements and they began the reduction of methylene blue at the top of the tubes. They produced less volatile acid than was produced by non-ropy strains. Stark[404] has noted a dissociation of certain bacteria into forms which produce ropiness in milk, and observed that such an effect may be induced in cultures by adding bacteriophage.

Cultures of bacteria of the lactobacillus group are frequently slimy but rarely are sufficiently tenacious to enable one to draw them out in threads. Ordinarily this sliminess disappears with thorough agitation. Some strains of *Sbm. casei* and of *Tbm. helveticum, bulgaricum* and *acidophilum* have been found to produce ropiness.[181] Buchanan and Hammer[50] cite a case in which ropiness was produced by the combined action of an organism of the *S. lactis* type with a bacillus having little or no effect on milk when grown alone. It is said that a mycoderm or an Oidium increases the tendency to sliminess when grown with *S. lactis,* and that this tendency varies among different lactic strains.[253] A mycoderm when grown with a lactobacillus produces a distinct ropiness in Swiss cheese cultures; but this condition seems to produce no detrimental effect in the cheese.

Rather few of the bacteria causing ropiness in milk are spore-formers, and pasteurization is usually efficient in checking an epidemic if subsequent contamination is avoided. Ropiness due to *B. lactis viscosus* and *E. neapolitana,* originating in contaminated water used for washing utensils, has been eliminated by pasteurizing the milk at 62.5° (145° F.) for 30 minutes.[361] Hammer and Hussong[166] report that *B. viscosum* is destroyed by pasteurization at 61.1° for 3 minutes. It has been shown that mature organisms causing ropiness may survive pasteurization at 62.8° for 30 minutes.[403] Bacteria grown under artificial conditions for test purposes are said to be less resistant in some cases than the same strains as they occur in nature;[403] moreover, old cultures, or organisms previously held at a low temperature, have been found to be more resistant than young strains,[166, 385] and some workers[385] have recommended holding the milk at about 15.5° for a time, rather than at a colder temperature, before pasteurizing. The contamination causing ropiness may occur after pasteurization.[166]

The cause of ropiness in milk has been traced to infected bedding, manure, stable dust or feeds,[403, 404] but it is frequently found to be in infected water in which cans or milk are placed to cool, or in which the

utensils are washed.[361, 404] Dusty feeds and improperly cleaned utensils are two chief sources of infection,[408] and water from polluted streams may be a source. After a time the bacteria may become so thoroughly disseminated about the dairy or milk plant that it is difficult to determine the original source. From the farm the infection may be carried to the factory or city milk plant and gradually spread to other farms. Imperfectly washed and sterilized cans may be the cause of this dissemination or it may result from infection of the outside of the cans from milk spilled on the floor of the receiving room or truck. These cans infect the water of the cooling tank, from which the infection easily finds its way into the milk.

The obvious methods of control include locating, if possible, the source of the infection, cleaning and disinfecting the floor and walls of infected stables, protecting the milk from the dust of the stable and farmyard, thorough washing and sterilizing of cans both inside and outside, cleaning the milk room and truck floors and disinfecting them with hypochlorite and sterilizing the cooling water with hypochlorite.

Bitter milk. Milk may become bitter through abnormal secretion especially when the cow is in an advanced stage of lactation. This condition is distinguished by the fact that the flavor is present when the milk is quite fresh and by a characteristic burning sensation in the back of the mouth when the milk is swallowed. Palmer[310] has shown that this condition is due to an abnormal amount of lipase in the milk which decomposes the fat with the liberation of butyric acid. Bailey[15] has described this defect as being due to lipase in the milk from old cows and cows late in the lactation period. A similar flavor may be produced in unpasteurized cream by the lipolytic variety of *Brucella abortus*. A salty-bitter flavor in milk may be due to bacterial infection of the udder.[231] When the bitter flavor is found in milk from cows having abnormal udder conditions or late in the lactation period, the sodium and chlorine content of the milk is usually high.[154]

Bitterness caused by the action of bacteria develops slowly and is more pronounced. Many of the bacteria causing this change are active casein digesters and some investigators have ascribed the bitter flavor to peptone. Lockhead[254] found that the flavor-producing constituent was non-volatile, seemed to have the complexity at least of an amino acid, and was probably a dipeptide. Trillat and Sauton[437] have shown in one case that bitterness was due to a combination of an aldehyde and ammonia which were formed simultaneously by a yeast and a bacterium.

Spore-formers are sometimes the cause of an intense bitterness in imperfectly sterilized evaporated milk; the bitterness may develop without other appreciable changes in the milk. Hammer[155] isolated an aerobic spore-former which slowly coagulated evaporated milk and imparted to it a characteristic bitter taste. Spitzer and Epplee[397] investigated an outbreak of bitterness in evaporated milk. The causative organism was a sporulating facultative aerobe similar to *B. panis*. Determinations of

nitrogen distribution in peptonized milk showed a large proportion of peptones to which apparently the bitterness was due.

Harrison [173] found that a torula was the cause of bitterness in milk and cheese. Hood and White [191] identified an atypical form of *E. coli* as the cause of a bitter flavor in milk. *Bact. fluorescens* has been found to cause a bitter taste in milk, and *Micr. pituitoparus* and *Bact. aerogenes* were found to cause a nauseous taste; [78] the probable origin of these organisms was the water used in washing the bottles and utensils. Newman [294] ascribed this defect to organisms of the Pseudomonas group. The organisms grew well at low temperatures, e.g., at 4°, and were found in the soil and in the wash water. Lockhead [254] found that bitterness in milk was caused by two Gram-negative, gelatin-liquefying, non-spore-forming, rod-shaped organisms. The defect was intensified when the two organisms were grown together, and the organisms were inhibited by *S. lactis*. The bitter flavor was most marked when the organisms were grown at a low temperature and when the air supply was increased. Some of the lactic streptococci have the ability to produce bitterness which is particularly evident when they are used in making cheese of the Neufchatel type.

Other abnormal flavors. The flavors of various feeds may enter the milk either through the animal body or by absorption from the atmosphere. Flavors from the juice of plants have been found to appear in the milk as early as 20 minutes after the plant food has been ingested, and the flavor in the milk may be pronounced within one hour.[342] Objectionable flavors from certain feeds can be at least partially controlled by feeding such feeds either a considerable time before milking or after milking is completed.[251] Certain foods, including good meadow grass, carrots, oats, rice meal and good quality hay are said to improve the flavor of milk.[251] Foods which may impart characteristic flavors to the milk include beets, potatoes, turnips and other root crops, certain types of silage, and pasture weeds such as wild onion, garlic, mustard, ragweed, cress, bitter-weed and chamomile. Post [320] stated that an offensive odor in milk and butter, prevalent in the fall months when beets are used as feed, is due to the presence of trimethylamine derived from betaine present in the beets; but Claasen [62] considers that it would not be possible for betaine to be changed to trimethylamine in normal metabolism.

Certain abnormal flavors may be due to digestive or reproductive disturbances of cows.[256, 79] Pathological udder conditions, including mammitis, garget and mastitis, may cause disagreeable flavors in milk.[256] Csiszár [79] noted a sweet-oily flavor which later changed to a sharp, rancid or even a soapy taste, and which was due to the presence of fat-splitting enzymes in the milk. Gondos [181] states that certain feeds cause a predisposition toward tallowiness in milk, and that among the factors contributing to the sweet tallowy taste are a high content of unsaturated fatty acids in the milk, the presence of oleinase, contamination by heavy metals which catalyze enzymic action and a lack of reducing substances in the milk. The tallowy flavor is due to the catalytic oxidation of unsaturated fatty

acids, and its development is activated by light, air, heat, milk enzymes and metals such as iron or copper.[436] A salty taste may occur late in lactation when the sugar content of the milk is low and the chloride content high,[341] or it may be due to udder inflammation. A rancid flavor due to lipase may occur several hours after milking.[341, 256] The development of rancidity is accelerated by homogenization.[103] The presence of metals such as iron, copper, tinned copper, monel metal, nickel, brass and bronze may produce, besides the metallic flavor, a subsequent catalytic decomposition of fat, resulting in oily, rancid, oxidized, tallowy, flat or cardboard flavors.[79, 181, 251, 267, 341, 358, 359]

Most of the feed flavors and many of the flavors mentioned above have been reported as also caused by the direct action of bacteria in milk. A flavor suggestive of *p*-cresol is said to be produced by certain thermoresistant spore-formers which are able to decompose tyrosine.[268] Certain peptonizing and liquefying organisms originating in manure are said to produce a barny flavor.[256] An aroma resembling amyl alcohol is said to be produced by certain white and orange micrococci similar to *M. caseolyticus* Evans.[80] The source of the aroma is thought to be leucine. A caramel flavor may result from the growth of certain strains of *S. lactis*.[212] Sadler [368] noted an undesirable flavor which was described as doughy and which was caused by strains of *Tetracoccus liquefaciens* Orla-Jensen. A disagreeable fruity flavor in condensed milk, accompanied by a coagulation, has been ascribed to an acid-producing, sucrose-fermenting coccus which gained entrance into the milk after it was condensed.[336] *B. ichthyosmius* is said to cause fishiness in milk, cream and evaporated milk.[153] A malt flavor may be caused by *S. lactis var. Maltigenes*,[160] and by certain micrococci of the aureus type.[485] Acid-formers retard the flavor development by these micrococci and *B. subtilis* intensifies it. Some of the *Actinomyces* cause a stale, musty or moldy taste.[116] A feed or stable flavor has been ascribed to an atypical strain of *Aerobacter oxytocum*.[364] A potato odor has been produced in cream by a rod-shaped, Gram-negative, aerobic organism.[47] The symbiotic action of a bacillus like *B. bulgaricus* and an alcohol-forming yeast produces a fruity odor in whey and cheese; the odor is said to be caused by acetaldehyde mixed with alcohol and acetic acid.[186] Prucha [321] and his associates studied an organism which caused a potato flavor; they have observed that some thermophilic organisms, which multiply rapidly in milk, produce off-flavors very quickly. *B. lactis foetidus*, a colon organism, produces a turnip odor and a bitter, repulsive taste.[251] Certain organisms which form hydrogen sulfide cause a putrid flavor.[306b] Peptonizing and liquefying bacteria originating in manure produce a barny flavor.[256] Certain strains of *S. lactis* produced a cabbagy [115] or a caramel [362] flavor, and *S. lactis* together with the aroma-forming organisms may produce a metallic flavor in buttermilk.[17] It is said that over-ripening a starter may cause a definite, undesirable flavor and aroma which seem to persist in subsequent transfers.[205] Lucas [256] and Ruehle [358, 359] have enumerated a large number of

flavors, mostly commonly associated with feeds, which are also caused by numerous organisms isolated from dairy products.

The development of flavors caused by microorganisms as well as those caused by enzymes can be most effectively arrested by efficient pasteurization of the milk, and the aeration which milk receives when it is run over a surface cooler helps to minimize undesirable odors.

Pigment formation. Pigments are excreted by many of the bacteria, yeasts and molds. These pigments occur in a great variety of colors covering the entire spectrum from the red of *Serratia marcescens* to the violet of *Chromobacterium violaceum.* Hammer [150] states that the production of color in milk by chromogenic bacteria depends in general upon the presence or absence of other organisms, the available oxygen, the temperature and the extent to which light is present. The color often forms first near the surface.[150, 405]

Water-soluble bacterial pigments are rare. Pyocyanin, formed by *Pseudomonas aeruginosa,* is an example of the few water-soluble pigments. More commonly they are insoluble in water and soluble in such fat solvents as ether, alcohol, acetone, chloroform, and pyridine. They are classed with the hydrocarbons or oxyhydrocarbons which have been described as luteins, lipochromes, lipoxanthines, chromolipoids, or carotenoids.[311] These include particularly the red, orange and yellow pigments although not all pigments of these colors are carotenoids. By determining the absorption bands of a bacterial pigment it can usually be separated into two and sometimes three or four distinct pigments.[245, 330]

Pigments are evidently not essential in the metabolism of the bacteria since the ability to produce them may be entirely suppressed without impairing the vigor of the culture. The pigment is excreted by the cell into the surrounding medium, while the organism itself may show no color.[150] Many species do not form pigment in the upper part of their temperature range and some are colored only on certain media or only in the presence of air. The ability to form pigment may be permanently lost by continued cultivation under unfavorable conditions.

The yellow pigments predominate among the chromogenic bacteria occurring in dairy products. They are especially common among the staphylococci and micrococci which come from the skin and udder of the cow. Many of these produce marked proteolysis. Two species of yellow pigment-producing bacilli were isolated from milk by Hammer.[152] One of these rapidly digested the casein while the other had little or no proteolytic action.

Bact. cyanogenes produces a blue color in milk but does not produce any other harmful changes.[150] A blue color has been found to be due to certain *Actinomyces;* [405] the causative compound resembled litmus in that the color changed to red when the reaction became acid. An *Oidium* has been reported as a cause of blue color in milk.[405] The blue and violet pigment formers are sometimes introduced into milk from water or soil but under modern conditions have little significance from an economic standpoint. When milk is set in shallow pans for 24 hours, a blue pig-

ment sometimes appears, but the short time that milk is now held and the insolubility of most of the bacterial pigments in water makes the pigment bacteria of minor significance.

Colonies of bacteria or yeasts sometimes make black, red or pink spots in sour milk or cream when it is allowed to stand. Bacterial pigmentation in cheese is discussed under abnormal fermentations of cheese.

Cultured milks. The effect of bacteria on the flavor and texture of milk was utilized in making special milk drinks many years before bacteria were seen by Van Leewenhoeck. Most of these fermented milks originated in the southern part of Russia and the countries around the eastern end of the Mediterranean Sea. They were made by producing conditions in milk favorable to the desired fermentation or by inoculating with small amounts of previously prepared milks. Usually the unwashed container was depended upon to provide the starter, especially when skin bottles were used. Under these conditions a mixture of bacteria was present and flavors were produced which are difficult to reproduce with pure cultures although the essential bacteria of these fermented milks are now known. In all cases a lactic fermentation is the basis, sometimes in combination with the production of gas, a mild alcoholic fermentation and an increase in the amount of soluble protein.[76] Yoghurt is said to contain about 0.20 per cent alcohol [76] and kefir may contain about 1.2 per cent.[88] Some fermented milks may be made from skim milk powder and water, and the body and texture of such a reconstructed milk can be improved by adding 0.35 per cent gelatin.[382]

One of the more popular fermented milks is known as yoghurt or yaourt in Bulgaria and Turkey, matzoon in Armenia, and leben in Egypt. In this milk the fermentation is usually brought about entirely by lactic acid bacteria, including *Tbm. yoghurt* (Orla-Jensen),[88] *L. bulgaricus,* an organism made famous by Metchnikoff as the bacillus of long life, and *S. thermophilus* (Orla-Jensen).[76, 88, 356] *S. lactis* (Lister) may be present but is not essential; *L. acidophilus* is sometimes added, in which case the product is called yoghurt-acidophilus milk. The fresh milk is pasteurized, cooled to 40° to 45°, inoculated, held at that temperature until ready for use, and is then put in a refrigerator.[88] Rosell [356] recommends an incubation temperature of 45° to 48°, at which temperature an acidity of 3.5 per cent develops in 24 to 36 hours. The body of yoghurt may be improved by heating the fresh milk at 90° for 5 minutes, homogenizing the milk after the culture is added, and adding 3 per cent of spray-dried milk powder.[272] Yoghurt is made from cow's milk, either pasteurized or partially evaporated, and in some places from sheep or buffalo milk.[356] The Egyptian leben is said frequently to contain a lactose-fermenting yeast which produces a mild alcoholic fermentation. Winckel [479] has described two new fermented milks called kajobst and kajovit which undergo a modified yoghurt fermentation.

Kefir is usually made from cow's milk and is peculiar in that the fermentation is brought about by kefir "grains" which resemble miniature cauliflowers. These "grains" consist of casein, yeasts and bacteria.[88] The

microorganisms used include lactose-fermenting Torula yeasts, *Str. lactis*, *Betabacterium caucasicum* and glycogen-containing rod-shaped kefir bacilli (Henneberg).[88, 318] The grains increase in size in the fermenting milk, and may be strained out of the milk, dried and kept for long periods for inoculating fresh batches of milk. The fermentation is usually carried on in closed bottles so that the gas is retained and the milk becomes effervescent.

In Russia a milk drink made from unpasteurized mare's milk and known as kumys is used extensively. The fermentation is due to *Bact. bulgaricum* principally, and also to lactose-fermenting Torula yeasts and *Bact. lactis acidi* Leichmann.[27] The milk is fermented in open vessels and the greater part of the gas escapes.

Kuban fermented milk, a product used in South Russia, is made from pasteurized milk by a combined lactic and alcoholic fermentation.[48] The micro-flora include a lactic streptococcus resembling *Str. hollandicus*, a lactic rod of the *L. bulgaricus* type, and three types of yeast.

Taette milk is a sour milk used in the Scandinavian peninsula. A slimy fermentation is induced by a variant of *Streptococcus lactis* to which the name *S. taette* has been given. This is possibly identical with *S. hollandicus* used as a starter in making Edam cheese, and with other streptococci causing ropy milk.

A milk drink known as saya is prepared from fresh unheated milk ripened at first by *S. lactis* and later by rod-shaped lactobacilli.[241] In saya milk, there occurs a considerable production of carbon dioxide and a strong proteolysis; it is characterized by the fact that it is ripened for six weeks at about 11°.

In a historical review of fermented milks, Corminboeuf[76] has described numerous other milk drinks, including mazun, gioddu, skorup and tättemjolk.

In making a commercial buttermilk with *L. bulgaricus* it is desirable to reduce the sharp acid flavor imparted by the bulgaricus culture by growing with it a streptococcus, but it is difficult to maintain the two in a mixed culture on account of the fact that the lactic streptococci cease growing at a pH of about 4.2 to 4.0 while bulgaricus carries the reaction to the neighborhood of pH 3.7 to 3.5. The same end may be accomplished by souring milk with the two cultures separately and then mixing them in the proper proportions to give the desired flavor and texture.

At one time much importance was attached to *L. bulgaricus* as a therapeutic agent. This was based on the work of Metchnikoff and some of his associates who, chiefly on clinical evidence, advanced the hypothesis that this organism could be established in the lower intestine where it suppressed bacteria of the putrefactive type and thus eliminated the injurious effects of their by-products. More recent work, particularly by Rettger and his associates,[384] has demonstrated that *L. bulgaricus* can not be implanted in the intestine but that this is the natural habitat of a closely related organism *L. acidophilus*.

Studies of *L. acidophilus* and *L. bulgaricus* by Weaver[468] suggest that

there is a definite relationship between implantability and the electrical charge carried by the organism. Recently *L. acidophilus* has been differentiated from other lactobacilli on the basis of the kind of lactic acid formed, temperature reactions, type of colony, fermentation of raffinose, mannitol and salicin, and inhibition by phenol and indol.[84] Only inactive lactic acid was produced by *L. acidophilus* while other members of the group produced more dextro than levo acid. Contrary to a belief formerly held, it is now apparent that the action of surface tension depressants cannot be used as a means of differentiating *L. acidophilus* and *L. bulgaricus,* according to Day and Gibbs,[93] and Curran, Rogers and Whittier.[84]

The bacterial flora of the intestine of the infant is confined almost exclusively to *L. acidophilus.* As the variety of foods increases the bacteria become more varied and, with a preponderance of proteolytic types, symptoms of autointoxication may develop.

In many well authenticated cases this condition is improved by the ingestion of milk cultures of *L. acidophilus* especially if the milk cultures are supplemented with liberal quantities of lactose. This sugar, unlike cane sugar and glucose, is not all digested in the upper part of the digestive system and is available in the lower intestine for fermentation by *L. acidophilus* which produces conditions unfavorable to the growth of putrefactive bacteria.[88] This result is probably due to an increase in the hydrogen-ion concentration with possibly some more direct biological effect.

To conserve the viability of the organisms the acidity of acidophilus milk should not be over 0.65 per cent, according to Kulp.[240] Others [89] state that the acidity should be developed to 1 per cent. Some workers [88] believe that the organisms retain their viability practically as well when the ripened milk is stored at 22° as they do at a lower temperature, while others state that the temperature should not be above 16°, preferably about 5°.[240] Kopeloff [284] and his coworkers have recently found that a "rough" strain of *L. acidophilus,* the only one of proven therapeutic value, lost its viability more rapidly at 4° than at 20°, while in the case of a "smooth" strain the reverse was true. They recommend keeping this fermented milk in a cool place but not in an ice-box. *L. acidophilus* cultures form acid rather slowly and are unable to overcome contamination by actively growing organisms, particularly those of anaerobic spore-forming type. Consequently, in preparing this culture for commercial purposes it is essential that the milk be completely sterilized. The drink may be prepared by inoculating evaporated milk which has been diluted with water.[387]

Flavors of butter. The flavor of butter is due primarily to the natural flavor of the cream from which it is made. Butter made from cream in which there has been no bacterial fermentation of any kind has a characteristic flavor, agreeable but mild. The nature of the flavor is dependent to some extent on the feed and may be affected unfavorably by the ingestion of highly flavored feeds of various kinds.

Fat is a solvent for a great number of flavor and aroma producing substances and the odors and flavors to which the milk or cream may be

exposed as well as those formed in the serum by bacteria, yeasts or molds may be carried into the butter.

A common practice in butter making is to increase the desirable flavor of the butter by inducing a bacterial fermentation in the cream. This may be accomplished by a spontaneous souring of unpasteurized cream; however, the quality and flavor are better controlled by adding to the pasteurized cream a culture starter of *S. lactis* or *S. cremoris,* usually containing certain associated aroma-forming organisms. The cream is often allowed to ripen for a few hours after the starter is added, or it may be churned immediately. The practice of making sweet cream butter from cream which has undergone little or no ripening is becoming more common.

There is a very appreciable difference in the flavor produced by different strains of the lactic streptococci. Pure cultures of *S. lactis,* forming chiefly lactic acid and only a small proportion of volatile acids, give milk an acid flavor. When they are used for ripening cream the butter lacks the desirable aroma characteristic of the best product.

Storch,[411] who was a pioneer in the study of butter cultures, used a pure culture which was later studied by Orla-Jensen [303] and named by him *Str. cremoris.* This culture is said to produce a typical butter aroma and to differ morphologically from *Str. lactis.* Conn, who was the first in this country to investigate the question of butter flavors, recognized that many cultures of *Str. lactis* gave only an acid flavor and that the typical butter flavor was probably not produced by a single organism. In 1919 Boekhout and Otto de Vries [41] isolated from a lactic starter a culture which, when grown with a true lactic streptococcus, produced a typical butter aroma. Orla-Jensen [303] considered this organism as being, like Storch's aroma producers, probably a weakened form of *Str. cremoris;* he and his associates [304] later indicated that the aroma organism probably belongs among the betacocci. Boekhout and Otto de Vries observed that when the aroma organism was grown with *S. lactis* the amount of volatile acid was increased. Hammer [157, 158] working independently, confirmed these observations and isolated two organisms which in conjunction with *Str. lactis* produced a typical butter aroma and improved the flavor of butter. The distinguishing characteristic of these cultures, to which he gave the names *Str. citrovorus* and *Str. paracitrovorus,* is their ability to produce volatile acids through the fermentation of citric acid. Other characteristics have been discussed under the lactic fermentation. Ayers and Mudge [18] isolated a similar culture from a commercial starter and, on account of its production of carbon dioxide, considered it as identical with *Str. kefir.* Hammer,[163] however, pointed out that there is no record of the fermentation of citrates by *Str. kefir* and reported the isolation from milk of a streptococcus which forms carbon dioxide without the fermentation of citrates, and Sherman [384] has shown that the origin of the carbon dioxide produced in milk by *Str. kefir* is not citrates. Orla-Jensen [304] questioned the significance of these cultures in the production of typical butter aroma and suggested that the aroma can be formed by *Str. cremoris,* particularly by weakened forms. He pointed out, however,

that both *Str. cremoris* and *Str. lactis* form *d*-lactic acid, while appreciable amounts of inactive acid as well as the *l*-form are found in good starters; the betacocci of Orla-Jensen, like Hammer's aroma bacteria, ferment citrates, produce large amounts of volatile acid, produce *l*-lactic acid, and are stimulated by the presence of yeast extract. Hammer has shown that a proper balance between *Str. lactis* and the associated organisms is necessary in a good starter, and that the addition of lactic acid or acids other than lactic stimulates the utilization of citric acid and the production of aroma. The so-called aroma bacteria have been variously classified under the generic names of *Streptococcus, Leuconostoc* and *Betacoccus.*

The first reference to the occurrence of biacetyl ($CH_3.CO.CO.CH_3$) and acetylmethylcarbinol ($CH_3.CHOH.CO.CH_3$), in dairy products is found in work of Schmalfuss and Barthmeyer,[369] who identified both substances in distillates from cultures of streptococci in milk. It had previously been known, however, that acetylmethylcarbinol is a product of bacterial metabolism.

Van Niel, Kluyver and Derx [282] isolated acetylmethylcarbinol from butter, and concluded that the characteristic aroma of butter is due to biacetyl. They pointed out that the biacetyl is formed from acetylmethylcarbinol by oxidation during cream ripening, and that the latter compound in pure form is odorless. These workers isolated acetylmethylcarbinol as an intermediate product in buttermilk and butter and in milk cultures in which *Str. cremoris, Str. citrovorus, Str. paracitrovorus* and some strains of propionic acid bacteria had been grown, but the tests in *Str. lactis* and *Str. thermophilus* cultures were negative. Schmalfuss and Barthmeyer stated that the presence of biacetyl can be detected by its aroma in a concentration of 0.00015 per cent, and van Niel and his associates stated that a butter of good aroma may contain about 0.0002 to 0.0004 per cent biacetyl. Other workers [427] have found approximately 0.0005 per cent biacetyl in fresh butter.

Kluyver [227] suggested that the role of the streptococci in cream ripening may be the production of enough acid to provide an hydrogen-ion concentration sufficiently high to enable the aroma bacteria to produce the aroma constituents from the citrates. This conclusion is partially a result of work of Hammer [271] and his associates in which they found that the aroma bacteria alone produce relatively large amounts of acetylmethylcarbinol and biacetyl if enough acid is added to produce a reaction represented by pH 4.3 to 4.0.

In cultures, the aroma compounds appear late in the ripening period, according to Hammer,[168] who stated that if the acidity is low, citric acid is changed chiefly into volatile acids, while if the acidity is high, relatively greater amounts of acetylmethylcarbinol and biacetyl are formed.

Tapernoux [419] found that the addition of biacetyl to butter or margarine produces a characteristic, desirable aroma, and that during storage the aroma disappears and tallowiness may occur. He believes that butter with considerable aroma or containing added biacetyl is relatively susceptible to the tallowy condition. Hammer [168] stated that the presence of

biacetyl in butter may increase the oxidation of oleic acid and cause bleaching and tallowiness. Wolff [485] has isolated an organism which he calls *Bact. diacetylum* (Voss), which is said to produce the butter-aroma constituents. The metabolic formation of acetylmethylcarbinol and its oxidation to biacetyl have been discussed under theories of fermentation (page 309). The question of aroma due to biacetyl in butter has been reviewed very comprehensively by Davies [92] and by Hammer and his associates.[168, 271]

The desirable flavor of butter once produced is not fixed, but changes more or less rapidly depending on a large number of factors. The flavor, aroma, and texture of butter are usually at their best just when it comes from the churn. If it has been made from cream of high quality, bacteriologically speaking, pasteurized under conditions which destroy bacteria without affecting the flavor of the cream materially, and ripened with a good starter, the deterioration of the butter may be slow, especially if it be held at very low temperatures. Even under the best conditions the butter loses its waxy texture and the flavor becomes less and less pleasing until a distinctly "off" flavor finally appears.

The nature of these off flavors varies greatly and they can not be accurately described. Butter held in cold storage which does not develop some of the more distinctive flavors, may have what is known as a storage flavor. An oily flavor is not uncommon; this varies from a taste suggestive of melted butterfat to one suggestive of machine oil. Metallic butter has much the same effect on the tongue as a piece of copper or rusty iron, and fishy butter suggests the presence of a trace of fish oil.

Tallowy butter, as its name implies, has the flavor and aroma of tallow and to the chemist the flavor suggests an oxidized fat. Cheesy butter has a flavor resembling cheese; rancid butter has the odor and flavor of butyric acid. Rancidity can be caused by *Ps. fluorescens.*[97] Fishy flavor is caused by small amounts of trimethylamine produced by the decomposition of lecithin.[87, 393, 413] Butter made from low acid cream is less likely to develop a fishy flavor than butter from high acid cream,[147] and excessive oxidation resulting from air incorporated in over-working the butter intensifies the defect. A burnt flavor has been ascribed to a heat-resistant organism which survived pasteurization and appeared to be a strain of *S. lactis,* although its sugar reactions resembled those of *S. thermophilus* (Orla-Jensen).[274] A fruity odor and flavor which occurred only in unsalted or lightly salted butter has been ascribed to a gelatin-liquefying organism tentatively identified as *B. punctatum.*[456]

So-called surface taint has been ascribed to *Achromobacter putrefaciens* [97] and to *Pseudomonas fluorescens.*[373] The development of surface taint is greatly inhibited by salt,[97] and occurs frequently in sweet or neutralized cream butter which has been contaminated by putrefactive organisms from impure water.[373]

In actual fact the flavor of butter, especially poor butter, is usually a composite result of two or more specific flavors which may be difficult to distinguish. In some cases a certain flavor may be that of a transition

stage between flavors. Thus an oily flavor is frequently a precursor of metallic flavor, and the metallic flavor may become fishy or tallowy. Very little is known about the nature of the substances causing the various metallic flavors. Spitzer and his coworkers,[398, 399, 400] in a study of the biological causes of deterioration of butter in storage, found that the acidity of butter should be within the limits represented by pH 5.0 to 6.0; if the butter is more alkaline than pH 6.0, the keeping quality is likely to be defective. They found that the decrease in quality of stored butter is proportional to the extent of protein hydrolysis. Of numerous proteolytic organisms studied, *B. ichthyosmius* and *B. proteus vulgaris* caused the greatest decrease in quality.

Aldehydes are probably among the important causes of "off" flavors. Some indication of this may be found in the completeness with which the flavor may be removed from butterfat by aeration, as is done in the renovating process. Rancidity is usually ascribed to butyric acid but is removed by aeration without materially reducing the free fatty acids.

Bacteria, yeasts, and molds, the inherent enzymes of the milk, and spontaneous chemical action have been suggested as possible causes of these changes in flavor. In considering the relative importance of these possible factors, the conditions under which changes in flavor take place should receive careful attention. Off flavors are not confined to butter made from poor materials or by careless methods. Butter which, in its fresh condition is of the highest quality, may develop the most pronounced metallic or fishy flavor. Low temperatures retard but do not prevent these changes. Any of the off flavors may appear in butter held at temperatures as low as 0° F. (— 16.6° C.), which is the usual temperature for long storage.

Enzymes can function, to some extent at least, under these conditions and in certain circumstances may be a factor in the deterioration of the butter.

Butter from unpasteurized cream has a tendency to become rancid or cheesy. In such changes the lipase and the proteolytic enzymes of milk could take part. However, the enzymes of milk are inactivated by pasteurization while this does not prevent changes in flavor.[347]

The conditions in the butter itself are not favorable to the growth of most bacteria. In addition to the unfavorable reaction and the relatively small amount of food material, the aqueous phase is a 14 or 15 per cent salt solution which is above the growth limits of all ordinary bacteria.

Under normal conditions there is no multiplication of bacteria in butter. The bacteria from the cream which are incorporated in the butter decrease at a rate dependent on the temperature at which the butter is held. Yeasts may multiply for a short time and some of the salt-tolerant bacteria may grow under some conditions, but it has never been shown that these organisms have any appreciable effect on the butter. Under certain unusual combinations of circumstances, as for instance heavy contamination, low salt and high temperature, ordinary bacteria may multiply in the

butter itself and produce disagreeable flavors. Eckles [107] has reported a case of putrid butter due to the development of proteolytic bacteria.

Flavors produced under these conditions, however, are not the ordinary off flavors of butter. It is very difficult to account for these changes by the direct action of bacteria when we take into consideration the development of off flavors at temperatures many degrees below the lower limits of bacterial activity. It is necessary, in order to explain changes in flavor under all the conditions specified, to assume that a certain amount of chemical change is due to the unstable nature of many of the constituents of butter. In the working and packing, air is incorporated in the butter in the proportion of about 10 per cent of its volume. In a short time the oxygen of this air disappears, possibly through oxidation of some of the unsaturated fatty acids. Moreover Holm [189] has shown that oxidation of fats may take place without free oxygen through a reaction in which it is probable that an intramolecular rearrangement takes place.

Without the accelerating action of the catalysts, the hydrolysis and the oxidations, which are probably the immediate causes of flavor development in storage butter, would be so slow that the effect would be inappreciable after a reasonable lapse of time. An acid reaction is essential to rapid change and it is now well known that the salts of such metals as iron, copper, and nickel have a marked effect in accelerating the development of off flavors, especially those of the oily, metallic, and fishy type.[348]

This explanation does not exclude bacteria as a factor in the deterioration of storage butter. It seems reasonable to suppose, however, that their action takes place in the milk and cream where they produce catalyzers or specific compounds which are later hydrolyzed or oxidized to flavoring substances. (See Chapter III.)

If these assumptions are correct it should be possible to make a butter of superior keeping quality by elimination, so far as possible, of cream in which bacterial action has taken place and which contains catalyzers from any source. Sweet cream butter made from pasteurized cream in which no acid has developed and which has not been exposed to rusty containers, worn pasteurizers, or other sources of metal salts, may be stored for long periods with very little change in flavor.

Abnormal canned milk. The treatment to which unsweetened condensed or evaporated milk is subjected during manufacture is such that the final product is usually sterile. The sterilization temperature of 115° for 10 minutes destroys all but the most resistant spores. For this reason all spoilage in this product caused by non-sporulating organisms may be attributed to defective containers or faulty sealing, either of which may permit the entrance and development of organisms after sterilization. Deming and Davis [95] examined 154 cans of unsweetened condensed milk purchased on the local market. The samples cultivated both aerobically and anaerobically at 37° and 55° yielded but one positive culture.

Some of the defects produced in this product by microorganisms have been discussed previously under bitter milk, pigment production, and abnormal flavors. In the so-called gassy fermentation, sufficient pressure

may be generated by internal evolution of gas to distort the container and often burst it along the seams. Organisms including aerobic and anaerobic spore-formers, micrococci and yeasts were isolated by Savage and Hunwicke [368] from blown cans of unsweetened condensed milk. The most frequent sources of this trouble are the highly resistant spores of both aerobes and anaerobes. The latter bring about a fermentation which produces butyric and other volatile acids and gas, which usually is a mixture of carbon dioxide and hydrogen. Putrefaction usually accompanies this kind of spoilage. An atypical form of *B. megatherium* was found by Kelly [211] to produce gas and coagulation. Infrequently streptococci may be the cause of gas and coagulation in evaporated milk, as shown by Hammer,[161] who isolated *S. distendens* from slightly blown tins.

The curdling of evaporated milk without gas production is a common cause of spoilage. In an outbreak of curdled evaporated milk, Hammer [151] isolated and described a sporulating facultative aerobe to which he gave the name *B. coagulans*. This organism appears to be a frequent cause of this kind of spoilage, as Hunziker and Cordes,[200] Cordes,[75] Hammer [167] and others have recovered organisms from coagulated evaporated milk whose characteristics corresponded to those of *B. coagulans*. According to Hammer,[167] *B. coagulans* increases both the total and volatile acidity and produces *d*-lactic acid; the volatile acids are acetic and propionic. Although there is no evidence of proteolysis, the organism greatly increases the soluble and amino nitrogen.

Hiscox and Christian [187] described a taint present in English commercial sterilized milk characterized by a strong and slightly acid smell reminiscent of the milk of coconuts, with an aftertaste described as "carbolic." The causative organism has some characteristics which resemble *B. novus*, but its identity was not definitely determined. Christian [61] found that periodic heating is essential for the completion of the life cycle of this organism. Spores in milk exposed to a temperature of 100° for 30 minutes germinate promptly and complete the spore cycle in about 3 days at 22°. In the absence of a high temperature, however, the spores do not germinate, due, it is believed, to the presence of a thermolabile inhibiting substance associated with the vegetative form.

In sweetened condensed milk the concentrations of milk solids and added sucrose are the principal factors which prevent its deterioration. It is rarely sterile and has a microflora quite different from that of evaporated milk. Cocci are usually present, and the growth of staphylococci seems to be especially favored. Spore-bearing aerobes, yeasts and coliform bacilli have been recorded frequently. Thermophiles were recovered from 76 per cent of tins by Savage and Hunwicke,[368] but they seemed to have little significance. Molds may cause spoilage under some conditions.

Gas formation in sweetened condensed milk is not uncommon. Pethybridge,[317] Hunziker,[199] Hammer,[156] Knudsen [228] and others have isolated budding organisms from blown tins. Rogers and Clemmer [349] isolated a coliform organism from blown cans of sweetened condensed milk.

Thickening is a common defect in sweetened condensed milk; Rice and

Downs[386] found this defect to be due to a sucrose-fermenting coccus which produced considerable acid. Downs[104] believed that most of the thickening by cocci is due to the formation of a rennin-like enzyme. The possibility that thickening may be a gelation process of physico-chemical nature should not be excluded.

Another defect sometimes encountered is the formation of small reddish-brown pieces of curd possessing a firm, cheesy consistency; these are referred to as "buttons." They usually float, and though they do not seriously affect the flavor, the defect is serious enough to cause the rejection of milk on the market. Rogers, Dahlberg and Evans reported that buttons are usually produced by the growth of a mold, *Aspergillus repens* (see Chap. XIII). The restriction of growth of the organism is due to the exhaustion of air in the can. These authors believe that button formation is caused by enzymatic action which is continued after the death of the mold.

Flavors of cheese. The ripening of cheese with its marked changes in physical properties, appearance, and flavor, is due to a combination of factors among which microorganisms are recognized as playing an important part. Many types of bacteria and molds are concerned in this ripening process. In each type of cheese there is usually a definite sequence of organisms which develop at successive stages of the ripening. Some of these organisms have little to do with the flavor production but are of importance in controlling the course of the fermentation and in preparing the ground for the bacteria or molds which give the cheese its characteristic flavor.

The nature of this biological sequence, and the resulting type of cheese, are determined in the making process, especially by the temperatures, the water retained, the amount of salt added and the method by which it is incorporated, and the temperature of curing. The methods by which bacteria are controlled have been developed empirically by the experience of generations of cheese makers, and from their experience there has resulted the highly specialized art of cheesemaking as it is practiced today. Thus it is possible to make from any milk of good quality cheese of such widely differing varieties as Cheddar, with its solid body and characteristic mild flavor; Swiss or Emmenthal, with its eyes, rubber-like texture and slightly sweet taste; and the soft-bodied, pungent Roquefort and Camembert cheeses.

Acid-flavor cheese. The simplest cheeses, both from the manufacturing and bacteriological point of view, are those of the cottage or Neufchatel type which undergo little or no ripening. The texture and flavor of these cheeses are due almost entirely to a lactic fermentation. The nature of this flavor may be varied within rather narrow limits by using starters of selected varieties of the lactic streptococci. The high acidity of the curd protects the cheese to a certain extent from the development of abnormal bacteria with consequent undesirable flavors. In cottage cheese the lactic fermentation may be followed by surface growth of oidium and similar organisms. A secondary development in Neufchatel

and cream cheese may be that of anaerobic spore-formers which give sour and bitter flavors.[467]

Hard rennet cheese. In hard cheeses, of which Cheddar, Edam, Emmenthal, and Parmesan cheeses are types, which undergo a definite ripening, a much more complicated curing process takes place. In the ripening of the cheese of this group two distinct processes are involved. One is a digestion of the curd in which a large part of the insoluble paracasein is converted into soluble products, among which are albumoses, peptone, amino acids, ammonia, and carbon dioxide. This digestion is brought about by a number of factors among which are the pepsin of the rennet, protease, the tryptic enzyme of the milk, and probably the lactic bacteria. This proteolysis has little direct effect on the characteristic flavor of the cheese and may be produced under conditions which do not permit the growth of bacteria. By this digestion the tough rubbery texture of the green Cheddar is converted into the soft waxy body possessed by the ripened cheese.

The digestion of the casein results in an accumulation of simpler nitrogen compounds, including histidine, tyrosine, guanidine, and lysine—in the older cheese putrescine is also found. In this decomposition of the casein molecule an appreciable amount of CO_2 is liberated and is given off in decreasing amounts as the cheese ripens.[489] In the later stages of ripening measurable quantities of ammonia are formed.

While a change in the proteins as profound as is indicated by the presence of these products can not take place without effect on the flavor, it is probable that the really characteristic flavors are produced in a secondary process in the ripening in which esters of the fatty acids are formed. The formation of esters goes on simultaneously with the protein decomposition but may be considered as the second step in the ripening. In the fermentation of lactose which is complete in from 3 to 6 days, lactic acid is formed in considerable quantity, and although there is probably a progressive change of lactic acid to other acids of the series, the total amount does not decrease, but may even increase as the ripening progresses. This additional lactic acid probably comes from the proteins.[416]

The lactates are converted to acetic and propionic acids and these in turn are broken down although acetic is at all stages the principal volatile acid. Butyric and caproic acids, evidently originating in the proteins or glycerine of the fat, accumulate slowly. Esters of these various acids are formed by combination with ethyl alcohol which probably has its origin in the lactates.

The characteristic flavors developed in the second stage of the ripening, are almost entirely of biological origin. The definite species of bacteria which produce these changes is still in doubt but the bacterial sequence, in Cheddar cheese at least, is fairly well established.

In making Cheddar cheese conditions are created which favor the development of the lactic streptococci, during both the making process and the early stages of the curing. The young cheeses contain this type of bacteria almost exclusively, and with the aid of the high buffering effect

of the milk solids which exist in the cheese in concentrated form, the lactose is completely fermented within a few days. In a normal cheese this is an acid fermentation without gas production.

In the making process the greater part of the bacteria is carried with the curd so that acid develops more rapidly in the curd than in the whey. The acid formed at this stage not only protects the cheese from abnormal fermentations but has a solvent action on the curd, making it more plastic so that it mats together when piled in the vat. The threads observed in the hot iron test are an indication of this change.

The lactic streptococci increase enormously in numbers in the first few days.[174] This type of bacteria decreases slowly as the ripening progresses. There is sometimes a large increase of liquefying cocci and always a development of the lactobacilli following the preliminary lactic fermentation. The latter group includes a number of varieties some of which produce levo, some dextro, and others inactive lactic acid.[175]

While Leitch [249] has shown that lactobacilli have an influence on the flavor of Cheddar cheese, it has not been demonstrated that they are responsible for the characteristic flavor.

In such cheeses as Swiss or Emmenthal the making process eliminates streptococci of the *S. lactis* type as important factors and encourages the lactic bacilli and *S. thermophilus* organisms which grow at relatively high temperatures.[125] The curd is heated in the kettle to a temperature so high (50° to 55°) that growth of nearly all bacteria is checked. *S. thermophilus* organisms usually increase in numbers, however, especially during the cooking. The lactobacilli and propionic acid organisms may decrease slightly in numbers during the making process. Gas-forming colon-aerogenes bacteria do not increase appreciably in numbers unless they are present in the milk in large numbers, in which case they may show a marked increase and cause a "pressler" cheese. The large mass of curd cools slowly in the hoops. Only organisms of *S. thermophilus* type grow actively for the first few hours, while the temperature is high; after a few hours the lactobacilli begin to multiply rapidly, completing within a few days the conversion of lactose into lactates. After removal from the press when one day old, the cheese is kept in a cold salt-brine tank for two or three days, and is held in a cold room for from ten days to two weeks; the low temperature greatly inhibits the initial lactic fermentation. The cheese is then transferred to a warm room where, as the pH increases, the growth of the eye-forming propionic acid bacteria is favored. This group plays a major part in the conversion of lactates into propionic and acetic acids by a reaction in which CO_2 is liberated. The making and curing processes produce an elastic curd considerably different from that of Cheddar cheese and the gas collects at foci throughout the cheese and forms eyes.

The gas in normal eyes of Emmenthal or Swiss cheese is principally carbon dioxide, and small amounts of nitrogen may be present.[64, 192, 193] The nitrogen is the residue of air occluded in the curd. Traces of hydrogen may be present as a result of abnormal fermentation. Clark [64] states that normal gas production occurs in two phases; first, a saturation with

carbon dioxide, and second, inflation of the eyes. Most of the carbon dioxide arises from the fermentation of the lactates. Clark compared the amount of carbon dioxide evolved with the total volatile acids produced and concluded that the activity of the propionic acid bacteria in Swiss cheese is not sufficient to account for all the carbon dioxide formed. Hostettler [192, 193] found that, besides the propionic acid organisms, other lactate-fermenting organisms promote gas formation even in the late fermentation when the eyes are being formed.

The changes in the protein follow much the same course as that of the changes taking place in Cheddar cheese,[481, 482] but the final flavor is quite different. The sweet flavor of the Emmenthal cheese develops simultaneously with the eyes and is probably due to the propionic acid bacteria. Bacteria of the *Lactobacillus casei* type are present in Emmenthal cheese in large numbers, but that they have an important rôle in the ripening has not been conclusively demonstrated.

Gruyère cheese is a hard cheese, very similar to Emmenthal, being made by the same process, which involves cooking and pressing of the curd. It differs from Emmenthal, however, in that a secondary fermentation takes place, originating in the moist smear on the surface and extending from the rind inward, with ammonia production and a softening of the curd. The ammonia is said to originate from the decomposition of intermediate protein decomposition products which have been formed in the fermentation by lactic organisms present in the curd; [96, 232] the secondary ammoniacal fermentation aids in giving to this cheese its characteristic flavor and aroma. Koestler [232] found the pH at the rind of ripened Gruyère cheese to be about 6.7 to 7.9 while the pH in the center was about 5.7 to 5.9. The organism chiefly responsible for this ripening is propagated in the washing water, and has been identified as *Bact. grueriense;* it is highly tolerant to salt, and acts on the cheese surface in symbiosis with certain other organisms, some of which are color-producers.[96]

The Parmesan cheese process follows in general that of Emmenthal but the fat and moisture are lower and the salt higher. There is the beginning of an eye formation, but at the end of the long curing period both the flavor and texture are in marked contrast to that of the Emmenthal. The identity of the bacteria responsible for these changes has not been established.

In cheese of the Limburger type more water is retained in the curd and a rapid and deep-seated splitting of the casein is obtained in which such highly flavored compounds as indol, skatol, and ammonia are found.

Mold-ripened cheese. Cheeses in which the characteristic flavor is obtained through the activity of molds, are of two types. In Camembert, Brie, and similar cheeses, the mold grows on the surface and the ripening progresses very distinctly from the outside toward the center. In Roquefort, Gorgonzola, and Stilton the mold grows in the interior and the ripening progresses more or less uniformly throughout the mass.

In all of these cheeses there are the preliminary lactic fermentation in

which the lactose is fermented, and changes in the proteins which follow the same general course as those taking place in the hard cheeses. Dox [105] has pointed out, however, that in Camembert the decomposition is of an ereptic nature rather than of the peptic type.

The molds are very active enzyme formers and produce a marked change in the outer layers of the curd.

Notwithstanding the partial liquefaction of the curd, indol, skatol, and other products characteristic of putrefaction are not formed. The acid reaction of the young cheese is reduced as the mold develops and bacterial growth is again resumed. It is not yet proved, however, that these bacteria have an important influence on flavor production. *Oidium* is present in all properly flavored cheese and may be a factor in the creation of the Camembert flavor which, apparently, the surface mold *Penicillium camemberti,* is incapable of producing alone.[428] In Roquefort the low temperature of the curing rooms (9°) and the high salt content (4 per cent) make conditions unfavorable for the growth of bacteria. Lactic streptococci are completely inhibited after the early stages of ripening, but other cocci, more tolerant to sodium chloride, and the lactobacilli develop to some extent. The bacterial content of Roquefort is much lower than that of Cheddar and Swiss and it is not probable that it has any essential part in the ripening beyond the initial lactic fermentation.[113]

To secure a normal Roquefort it is essential that conditions be produced which will promote a fairly abundant and well distributed growth of the Roquefort mold, *Penicillium roqueforti.* This mold is peculiar in that it will grow under a lower oxygen tension than most molds. By punching small holes in the cheese at the right stage of development, sufficient air is admitted to maintain an atmosphere in the interior of the cheese containing 2.0 to 7.0 per cent oxygen. Under these conditions the Roquefort mold sends its mycelia into every crevice of the curd and gives the cheese its mottled appearance and pungent flavor.[429] The sharp, peppery flavor of Roquefort is due, as Currie has shown,[86] to the liberation of a lipase by the mold which hydrolyzes the fat with a gradual accumulation of fatty acids. Of these acids capric, caprylic, and caproic have a peppery taste and are responsible for the characteristic flavor of Roquefort.

Abnormal fermentations of cheese. The value of cheese may be affected by abnormal fermentations which change the flavor, the odor, or general appearance of the cheese mass. These fermentations may be due to an unusual contamination of the milk, but more frequently they are caused by bacteria normally present in the milk which are enabled to develop through some combination of circumstances which disturbs the biological balance existing in the normal cheese. In cheese of the Neufchatel type bitter flavors may be produced by certain strains of the lactic streptococci but the sour, bitter flavor which develops when the cheese is several days old is due to the active production of volatile acids by anaerobic spore-formers. An unclean odor, accompanied by gas, in milk set for cottage cheese has been caused by an Escherichia-Aerobacter organism.[367] "Stinker" spots in Emmenthal cheese are caused by *Cl. putrifi-*

cum.[407, 7] The elimination of the "stinker" fermentation by the use of an active lactobacillus starter shows the necessity of establishing a dominance of the lactobacillus group. Stocker[407] noted so-called foul spots in Emmenthàl cheese and stated that proteolytic cocci were the chief cause, although yeasts were also present.

Gassy fermentations in Swiss cheese are known as "nissler" or "pressler," depending on the rapidity and extent of the fermentation. A "nissler" is characterized by a uniform distribution of small eyes or "pinholes" throughout all or a large area of the cheese; this condition may occur late, often after several weeks. A "pressler" cheese is one in which the gassy fermentation appears in the cheese on the press. The gas holes are often larger and in most cases confined to an area near the surface. Gassy fermentations which take place in the vat or while the cheese is still in the press are caused usually by the colon-aerogenes group. These bacteria are active lactose fermenters and are not easily inhibited by the lactic fermentation. However, they have little effect in cheese after the lactose has been fermented. Abnormal gassy fermentations which appear later in the cheese, especially some of the "nissler" fermentations of Swiss cheese, are usually caused by anaerobic spore-formers of the *C. welchii* type.

Albus found that a strain of *C. welchii* caused an abnormal gassy fermentation in Swiss cheese,[4] but that some unknown factors other than the presence of the gas-producing organism and the present method of manufacture play a part in the occurrence of the gassy defect. He stated that the use of *L. bulgaricus* together with a high cooking temperature had little or no effect in suppressing this organism, but that this method is efficacious in suppressing growth of members of the colon-aerogenes group. Numerous experiments have shown that conditions for a gassy fermentation depend partially upon the number of gas-forming organisms in relation to the number of true lactic acid producers.[148, 409] When insufficient starter is used, acidity develops slowly, the curd remains soft and spongy, and the tendency toward gassiness is increased; an excess of starter, however, may injure the texture by producing an excessively short, friable curd.[148] Gassy cheese may result from stringy milk due to *A. aerogenes*,[7, 409] or from alcohol-producing yeasts, which also produce an undesirable flavor.[409] "Blowing" on the press is usually caused by coli or aerogenes organisms; "blowing" in the warm room or ripening cellar is often caused by *B. amylobacter*.[7, 141] Haglund and his coworkers[148] stated that *B. coli* is of much less importance than *A. aerogenes* in causing gassy cheese, and that the aerogenes organisms originate largely from animal feces. Orla-Jensen,[268] Barthel[21] and others[192, 193] have shown that butyric acid bacilli may cause an abnormal gassy fermentation. Gassiness from butyric acid organisms may occur late in the ripening, even when an early gassiness due to the coli-aerogenes group has been suppressed by the use of starters or by other means.[408] European workers[87, 101] have reported that silage is often a source of gas-forming anaerobic spore-formers. Most of the silage with which they worked was

grass silage which was much more heavily contaminated than corn silage.[101, 239] Gassy spots or "blow-holes" in Swiss cheese may be caused by an abnormal fermentation arising in an area where there is an excess of fine particles or "cheese-powder," or a whey pocket.[233] The gas in these abnormal spots is said to contain mostly carbon dioxide, as in the case of normal eyes. Rennet cheese is more subject to gassy fermentations than sour-milk cheese because the former contains a smaller number of lactic acid organisms.[409] Gassiness in processed cheese is commonly caused by *B. amylobacter,* members of the sporogenes group, certain strains of *B. putrificus* and butyric acid bacteria; butyric or putrefactive flavors often accompany the gassy defect.[142] Increasing the acidity to pH 5.0, together with efficient pasteurization, are corrective measures. Gassy fermentations are sometimes caused by lactose-fermenting yeasts which become established in the whey vats and find their way into the milk through the farmers' milk cans. The abnormal gassy fermentations not only injure the appearance of the cheese, but they are usually accompanied by changes in flavor of an objectionable nature.

The gas formed in abnormal fermentations is largely hydrogen.[64, 148] Hostettler[198] found only 0.3 per cent hydrogen in the gas in a normal cheese, and as high as 64 per cent hydrogen in the gas in a typical "pressler." The hydrogen is probably formed from the decomposition of formic acid.

Aside from the influences mentioned above, certain workers claim that some milks have an inherent tendency, due to unknown chemical or physico-chemical factors in the milks, to undergo abnormal fermentations or to act abnormally. This tendency is referred to by Huttig[197] and others as the "disposition" of the milk. Some milks have a greater tendency than others to produce gassy cheese when *B. aerogenes* or other gas-producing organisms are present. Such a milk, like that produced by cows having mastitis, is in many cases curdled only slowly by rennet.

The use of saltpeter has recently been found effective, according to several workers,[148, 408, 454, 483] in controlling gassy fermentations. A concentration of potassium nitrate as low as 0.02 per cent is said to be effective as an inhibitor of coli-aerogenes and other gas-forming organisms. Some workers[408] state that this treatment is detrimental to the flavor and causes discoloration in cheese; the method has not met with any wide application.

It is not always possible to control these organisms by the usual development of acid by lactobacilli starters. Since it is evident that factors other than acidity are active, it is desirable to adjust the starter to secure a prompt return to multiplication by the starter bacteria when temperature conditions become favorable, and consequently to secure the development of a large number of lactobacillus cells as early as possible.

Pigment formation or discoloration of cheese by microorganisms is usually the result of improper care in the curing room or contamination due to rind defects. Blackening of the surface may be caused by molds, especially *Monilia nigra,*[7, 406, 410] and is often accompanied by rind decay.

A similar discoloration is produced by a mold of the *Cladosporium* type and also by a non-motile aerobic bacterium which grows well at low temperatures.[410] *Penicillium casei* produces brown rind spots.[7] Mold discoloration is usually due to surface organisms rather than to those present in the milk and commonly occurs in rind cracks or trier holes from which the defect penetrates into the curd.[276] Reddish spots and rind decay on hard cheese may be caused by a mold, *Oospora aurantiaca*.[410] Reddish spots on Camembert or Limburger cheese, accompanied by an undesirable flavor, have been ascribed to *Micrococcus roseus*.[410] A red color and a bitter taste, accompanied by blisters on the rind, have been observed in fromage de Comté, and are attributed to the presence of nitrates from the walls of the caves or from the soil. The nitrates penetrate into the curd and the red color is said to occur when the nitrates are reduced to nitrites by the action of surface organisms.[210]

Oospora caseivorans causes rind bleaching and decay.[7] Dark spots on or immediately under the rind in Emmenthal cheese may be caused by *B. Güntheri* Lehm.[430] Surface defects are largely eliminated by proper care in washing and salting. In extreme cases, a milk of lime solution containing one per cent formalin has been recommended.[410]

Discoloration of the curd may be caused by mold enzymes diffusing into the cheese. Brown and red spots in Emmenthal cheese have been found to be caused by strains of *B. acidi propionici*.[430] Orange to red spots in the curd of English hard cheese have been found to be caused by a lactic organism resembling Orla-Jensen's *Betabacterium*.[90] Rusty spots in Cheddar cheese have been caused by strains of anaerobic lactic acid bacteria, the growth of which seemed to be stimulated by certain other organisms which produced a low oxidation-reduction potential.[91] Certain workers observed reddish lines or veins in Gouda cheese caused by a non-liquefying rod-shaped lactic acid organism which produced pigment only when grown in the absence of oxygen.[36] A muddy or bleached discoloration in Cheddar cheese has been caused by molds which gain entrance through rind cracks.[276] A pink discoloration or a mottled condition may be due to reduction of the annatto color by molds or other microorganisms.[276, 488] Bacterial enzymes are said to reduce the lead salts present in the annatto coloring material.[275] Bleaching or mottling may be due to fat decomposition by bacteria or molds.[276, 488]

When the curd is uniformly darkened, the defect may be due to the presence of traces of copper or iron from untinned cheese kettles or other equipment.[410] Tin, antimony or lead dissolved from tin foil wrappers has been found to cause a surface darkening when the acidity is high and when excessive moisture is present.[128] Zinc foil may be used to correct this defect.[307] Black spots in cheese may be caused by the presence of lead from paint, pipes, or vats.[250]

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Chapter XII

Influence of Physical and Chemical Factors on Bacterial Growth

Influence of Hydrogen-Ion Concentration

The growth and multiplication of microorganisms and the rate and nature of their metabolism are dependent on other factors besides the mere presence of suitable nutritive material. The physico-chemical equilibria of the medium must necessarily govern all the phenomena which take place at the cell surface, and hence control the possibility of assimilation and excretion, the states of hydration of the materials in the protoplasm and consequently the very life of the cell. In preparing a medium for bacteria, or in considering the possibility of bacterial growth in or on a certain substance, it must be remembered that the medium represents both food and environment. Most bacteria are capable of adapting themselves to environmental changes, but only within a fairly well defined range of temperature, surface tension, hydrogen-ion concentration and osmotic pressure. In studying the influence of any one of these factors, with the others held constant, there may be observed an optimum point or zone and a more or less extended growth range outside the limits of which multiplication is inhibited.

Pasteur and his contemporaries recognized the importance of the "reaction" of the medium although determinations of titratable acidities gave very inconsistent data. Except in the case of certain standard media made from uniform ingredients, the best results were often attained by methods which appeared somewhat casual, such as adjustment to "neutrality" with litmus rather than by actual titration. Modern methods of hydrogen-ion determination have cleared away many of these apparent discrepancies, and it becomes obvious that it is the hydrogen-ion concentration of the medium which must be adjusted within certain limits rather than the ability of the medium to neutralize standard alkali. The hydrogen-ion concentration depends upon the kinds of acids present, their degree of dissociation, and the amounts and kinds of buffers present. Two media with the same titratable acidities may have widely different pH values depending on the buffer action of the salts and proteins present. These considerations are discussed in detail by Clark [88] who gives a very comprehensive bibliography. Here it will be sufficient to allude briefly to certain investigations of particular interest without attempting an exhaustive or historical treatment of the subject.

Studies of the relations of hydrogen-ion concentration and bacteria have taken two lines—determination of the ranges favorable to growth for specific organisms, and determination of the characteristic final hydrogen-ion concentration attained by cultures. Dernby [53] recorded the growth-range and the optimum on sugar-free broth, for a large number of organisms, and on that basis classified them into two groups. The first group which can tolerate a wide range of pH includes such organisms as *B. subtilis* (4.5 to 8.5, optimum 6.0 to 7.5), *B. proteus* (4.4 to 8.4, optimum 6.0 to 7.0), and certain anaerobes (5.8 to 8.5, optimum 6.0 to 7.6). The other group was limited to a much narrower range, and includes many of the more important pathogenic organisms such as *B. typhosus* (6.2 to 7.6, optimum 6.8 to 7.2), *B. diphtheriae* (6.0 to 8.3, optimum 7.3 to 7.6), *Pneumococcus* (7.0 to 8.3, optimum 7.8), and *B. tuberculosis* (6.0 to 7.6, optimum 6.8 to 7.2). Virtanen and his coworkers [164, 165] found the optimum pH for *S. lactis* and *B. casei* ε to be 6.2. Growth of *B. casei* ε stopped at pH 4.9. At pH 7.2 the growth of *B. casei* ε was very slow but the fermentation continued at a rate three-fourths as great as at the optimum pH. A careful quantitative study by Cohen and Clark [40] on *B. coli* and *B. bulgaricus* in the logarithmic phase of growth shows clearly that there is a fairly broad pH range through which the rate of multiplication is about the same, but that at its borders slight changes produce marked effects. The optimum range appears somewhat narrower if the entire growth curve is observed. The lag period is noticeably longer in alkaline media, and the organisms are more sensitive to heat at the acid end of the growth range. Study of the literature makes it obvious that for a given bacterium the exact value and spread of the optimum range for growth will vary with the temperature of incubation and with variations in the medium, such as salt content, buffer value, the nature and amount of protein available, and the presence of sugars attacked by the organism. The final hydrogen-ion concentration attained is even more subject to these influences and is also affected by the initial reaction of the medium.

Clark [37] and Cohen and Clark [40] have observed that in a highly buffered medium the final pH attained by *B. coli* is lower than in one less buffered, and that the acid border of the growth range in *B. coli* and *B. bulgaricus* shifts with the nature of the acid. Evidently there are other factors at work beside the simple physical effects of ionic activity. The studies of Van Dam [162] and Holwerda [84] make it clear that the self-limitation of lactic acid cultures is due not to the actual ionic concentration, but to the accumulation of undissociated lactic acid. Kolthoff [97] points out that to define precisely the point at which growth ceases on a given medium one must know both the pH and the nature and amount of acid produced. Rogers and Whittier [182] have shown that the growth of *S. lactis* in milk is limited not only by the concentration of hydrogen ions but also by the concentration of undissociated lactic acid. When sodium lactate was added, the limiting pH, which was ordinarily 4.25 in milk, increased to a higher value depending upon the amount of lactate added. The greatest cell populations were attained in milk when the pH was controlled at 5.8 to 6.0. In arti-

ficially prepared media acetic acid causes growth to cease at a lower hydrogen-ion concentration than does lactic acid, while addition of hydrochloric acid does not greatly affect the final pH—the inhibitory effect seems to vary inversely with the dissociation constant of the acid.

The presence of certain salts may affect the sensitiveness of an organism toward changes in the reaction. The survival of *B. coli* in water has been found to be greater at pH 6.0 than at pH 5.5 or 7.0; but when $CaCl_2$ was added, the point of maximum survival was shifted toward pH 5.0.[158, 178] Sherman and Holm[145] found that the pH range for optimum growth of *B. coli* in peptone plus dilute sodium citrate was narrower than in peptone alone; when *B. coli* or *B. alcaligines* was grown in peptone plus dilute sodium chloride the pH range for optimum growth was wider than when the organism was grown in peptone alone. These workers observed that a salt which decreases the rate of growth also narrows the limits of hydrogen-ion concentration at which *B. coli* will grow.

Winslow and Falk[178] found that in an alkaline solution a strain of *B. coli* had the power to alter the reaction of the medium so as to decrease the alkalinity markedly, a process which was inhibited by $CaCl_2$. Others[158] state that *B. coli* is able to change the pH of the medium toward its optimum by excreting from the cell either ammonia or carbon dioxide, according as the reaction of the medium is on the acid or the alkaline side of the optimum, the process terminating at pH 6.2 to 6.4.

In the case of certain propionic acid bacteria which are said to be largely responsible for the production of eyes and the characteristic, sweetish flavor in Swiss cheese, Sherman[144] has shown that these organisms grow better in a broth containing sodium lactate at pH 7.2, than in a calcium lactate broth at pH 5.2. In the ripening of Swiss cheese, the activity of these organisms, as manifested in the formation of eyes, is not apparent in the early ripening at pH 5.0 to 5.25, but becomes pronounced in the later ripening when the pH reaches about 5.5 to 5.8.

When it is desired to minimize the inhibitory effect of hydrogen ions, in studying the growth and activity of bacteria, sterile chalk may be added to the medium. In milk buffered with an excess of chalk (40 gms. $CaCO_3$ per liter), such organisms as *S. thermophilus* or the lactobacilli have been found to increase the hydrogen-ion concentration only to pH 5.2 to 4.9.[185]

Lactic acid-producing starters, used in the manufacture of cheese, greatly improve the quality of the finished product. European workers attribute this principally to their effect on the ripening process. The inhibitory effect which such a massive inoculation must have on the undesirable organisms present in the milk is doubtless an important factor. A complete picture of the relationships involved should include also the large amount of undissociated lactic acid formed and the low pH which, while not necessarily inhibitory for gas-producing or putrefactive organisms, is nevertheless somewhat below their optimum range. However, the hydrogen-ion concentrations are only inhibitory, and are not lethal except for very sensitive strains. Thus Wedemann[169] found that *B. tuberculosis*

survived for 18 days in milk of high acidity and bacteria of the typhoid-enteriditis group for an even longer time.

Results of considerable theoretical and practical importance have been obtained by observing the final pH in cultures. While the precise concentration attained is dependent to some extent on the nature of the food supply, in a suitable well-buffered medium of standard composition, the final hydrogen-ion concentration of a pure culture is remarkably constant and is characteristic of the organism in question. This value should not be confused with the inhibitory concentration, for in many instances other chemical and physical factors may cause growth or fermentation to cease before the inhibitory pH is reached. The self-regulation of the coli-aerogenes group is an excellent example. In a medium containing 0.5 per cent dextrose, 0.5 per cent peptone and 0.5 per cent K_2HPO_4, *Escherichia coli* reaches a pH of about 5.0, whereupon growth ceases. *Aerobacter aerogenes* presents a very different picture. The organic acids resulting from the fermentation of the dextrose are in turn fermented to carbonates. Thus the pH observed during vigorous growth is the resultant of two opposing fermentations, and the sugar is exhausted before an inhibitory concentration is reached. After the culture is sugar-free there is a considerable reversion toward alkalinity. The practical result of studies of these phenomena is the methyl red test of Clark and Lubs, of great value in distinguishing strains of fecal origin from those characteristically found on plants.

The final hydrogen-ion concentration is also of value as a means of distinguishing potentially pathogenic streptococci from harmless bovine strains. Ayers [10, 14] observed that, in a yeast infusion medium, strains from human infections produced final hydrogen-ion concentrations between pH 5.5 and pH 6.0, while strains isolated from the udders, feces, and mouths of cows attained a value of 4.6 to 4.8. This observation was confirmed and elaborated by Avery and Cullen [4] and others. While the absolute values depend somewhat upon the nature of the medium, the strains from human sources are checked at a hydrogen-ion concentration much lower than those observed in cultures from bovine sources.

Some organisms may show radical morphological changes, depending upon whether they are grown in an acid or in an alkaline medium.[57]

The importance of hydrogen-ion concentration in influencing the action of many substances used as preservatives has been shown by Cruess and Rickert,[44] Behre,[26] Cruess,[45] and other workers. The latter investigator observed that at neutrality approximately 4 per cent of sodium benzoate was required to prevent the growth of most fermentation microorganisms studied, whereas at pH 2.3 to 2.4 only 0.02 to 0.03 per cent was required and at pH 3.5 to 4.0 (the range for most fruit juices) there was required 0.06 to 0.10 per cent for the fermentation organisms. It is probable, however, that the undissociated acids rather than their ions are the preservative agents in the presence of most organic acids.

Hydrogen-ion concentration is doubtless one of the chief determining factors in the well-known succession of flora in unheated milk. Fresh

milk secreted at pH about 6.60 is a favorable medium for most bacteria. As long as available sugar remains, the lactose-fermenting types usually predominate. Ultimately their multiplication ceases. The hydrogen-ion concentration at this juncture, usually between pH 4.0 and 5.0, while decidedly unfavorable to bacteria, is not lethal and does not at all inhibit the growth of molds and yeasts. Moreover, many molds are capable of using lactic acid as a source of carbon. There ensues a period of mold development. The combined result of the destruction of lactic acid, and the liberation of weaker acids, ammonia and alkaline products of protein decomposition gradually brings the reaction back toward neutrality. From this time on, conditions are favorable for the characteristically putrefactive bacteria, and the final period of protein decomposition is predominantly bacterial. The organisms concerned in these successive fermentations are discussed on page 356.

Influence of Temperature

General. Temperature is one of the important physical factors, undoubtedly the most important one, which influences the life of microorganisms. The relations of the bacterial cell to temperature are expressed by specifying three points on the thermometric scale, the minimum, the optimum and the maximum. The minimum point is the lowest temperature at which the slightest growth can be observed. The optimum is that temperature at which the different cell functions are best performed, that is, where such things as cell reproduction and fermentative power are at a maximum. The optimum temperature for growth, however, does not always coincide with the optimum temperature for fermentation. The maximum is that temperature which can not be exceeded without cessation of growth. The majority of bacteria can not exercise normal metabolism except in the range of temperature from about 6° to 45° C. (42.8° to 113° F.). Exceptions will be discussed later.

The temperature at which milk or one of its by-products is held influences not only the rate of growth of organisms in the milk but also favors or inhibits the growth of the various organisms. With variations in incubation temperature the amount and kind of fermentation may vary considerably. Usually the fermentation is typical for a given temperature as that temperature favors a certain kind of organism or a succession of organisms, and it is sometimes possible to predict the cycle of fermentations through which milk will go at a given temperature. The proper temperature of incubation is of great importance in maintaining the proper proportions of organisms in a mixed culture of starter bacteria and a good starter can sometimes be prepared by holding milk at a definite temperature. One type of commercial buttermilk has been prepared in this way. The temperature of incubation will usually favor organisms whose optimum temperature is near that point, but in some cases the temperature may be less unfavorable to one of a group of organisms present and may favor its growth. An example is the development of ropiness in milk

held at about 10° C. (50° F.). The optimum temperature for the ropy milk organism is about 20° C. (68° F.), but it has less competition from *S. lactis* at the lower temperature.

In order to understand clearly the influence of temperature, it must be realized that it is related not only to growth of the cell but to its death as well. When the maximum temperature for growth is exceeded, injury to the bacterial cell results and the cell dies. This temperature is known as the thermal death point of the organism. Growth at the maximum temperature, in the case of some bacteria whose thermal death point may not be far from the maximum temperature, results in the rather rapid destruction of the bacterial cell, although in such cases death is not instantaneous but proceeds at a rate more or less predictable. This has been explained as an outcome of accelerated metabolism which leads to autodestruction. When there is a rapid increase in temperature the degree of heat accelerates death so that it appears immediately. The destruction of bacteria by relatively rapid heating to high temperatures in the region of the thermal death point of the organism should not be confused with the death of the cell by holding for long periods at temperatures just above the maximum for growth. While the mechanism of the cell destruction may be similar if not identical, nevertheless, there are practical applications of the thermal death point which make it advisable to keep the two death processes distinct for purposes of discussion.

Optimum temperatures for many of the milk organisms are given below and will indicate the type of growth favored by different temperatures. Here only a brief discussion will be given of the fermentations to be expected in milk held at various temperatures. At temperatures below freezing bacteria do not multiply and gradually decrease in numbers. Fermentation will sometimes take place slowly, however, at temperatures below the minimum for growth. Runow [134] reports a continuation of the lactic fermentation under such conditions, but due entirely to living cells. At temperatures just above freezing and up to about 5° C. (41° F.) there is usually a decrease in the number of bacteria in milk, followed by a slow increase in numbers for several weeks up to very large numbers. Little change in flavor may be observed, but usually undesirable flavors and odors develop, with proteolysis and putrefaction, but little or no acid formation.[116, 117, 125, 41, 71] It is difficult to predict the changes which will take place in milk held at about 10° C. (50° F.); they will vary with the kind of organisms present and their proportion. Action may be similar to that at 5° C. (41° F.) but more rapid, a slow acid fermentation may take place, or a bitter, ropy or other abnormal fermentation may occur. A temperature of about 20° C. (68° F.) will usually favor a lactic acid fermentation by bacteria of the *S. lactis* type. In the absence of these bacteria, *Aerobacter aerogenes,* or closely related organisms may cause a gassy, acid fermentation with undesirable flavors and odors, or yeasts may produce off flavors. At 30° C. (80° F.) most common milk organisms can grow, but the *S. lactis* type will usually predominate if present in any number, and bacteria of the Escherichia-Aerobacter group will

grow and produce gas and off flavors unless too greatly outnumbered by *S. lactis*. In milk held at 37° C. (98.0° F.) acid development is usually rapid due to growth of *S. lactis*, but conditions are also favorable to gas-forming bacteria of the Escherichia-Aerobacter group and they usually grow considerably at this temperature. Many of the lactobacilli are also favored by this temperature and develop sooner or later in the milk with high acidity as the result. Anaerobic gas-forming bacteria are also favored and blow the curd apart if these bacteria were present in considerable numbers in the original milk. *S. lactis* is often low in numbers or absent in milk produced by modern methods, and bacteria of the *S. thermophilus* type carry on the acid fermentation. In milk held at temperatures above 40° C. (104° F.) organisms like *S. thermophilus* or *S. fecalis* and lactobacilli are favored and may predominate. *S. thermophilus* would usually predominate in the earlier hours of growth. At temperatures of 52° to 55° C. (125° to 131° F.), or above, only truly thermophilic bacteria grow, usually of the *Lactobacillus thermophilus* [22] type.

Retarding effect of low temperature. It can not be said that low temperatures show the same definite relation to the thermal death point as do the high temperatures. Experiments [125] using low temperatures obtained by liquid air and liquid hydrogen indicate clearly that cold can not be depended upon to destroy different species of bacteria and that even different races [139] show a marked variability in resistance to low temperatures. There is, generally speaking, a slow destruction of bacteria when they are held below the freezing point, a destruction which continues for weeks and even months. The action of cold is directly opposite to that of heat in that cell activity is at its lowest point at low temperatures. Theoretically, the critical survival point should be just below the minimum growth temperatures, but new factors enter into the problem at this point.

It is logical to believe that at temperatures below the freezing point the mechanical effects on the bacterial cell, causing rupture by internal pressure or by external pressure developed during crystallization, should add their effects to the suspension of cell functions. The greater destructive effect of solid freezing [95] in water at —20° C. (—4.0° F.) upon *Escherichia coli* as compared with the same temperature in mixtures of water and glycerine, are suggestive. With *E. coli* in water and glycerine at —20° C. (—4.0° F.) a large percentage of the cells remained alive for six months. Earlier investigations [128] led to the conclusion that the killing action of cold is the most important factor in cell destruction. However, later studies [79] indicate clearly that the degree of cold below the freezing point is not a very important factor in the destruction of bacteria. There appears to be no critical temperature below freezing where the germicidal effect is greatly accelerated. These experiments indicated that crystallization, probably resulting in mechanical crushing, is an important germicidal factor in causing the death of bacteria at 0° C. and below. It was also found that the reduction in numbers by freezing was much less in milk and cream than in pure tap water. More recent studies [140] emphasize

the importance of the nature of the solution or medium in relation to low temperatures and lay stress on the mechanical protection given bacteria by sheering ice crystals as well as various solutes.

With milk and other dairy products one usually is not concerned with the effect of temperatures below the freezing point except in the case of ice cream. Here the mix is not only subjected to a freezing temperature but the ice cream may be held for considerable periods at low temperatures in hardening rooms. Various studies [11, 60, 72] have failed to show any indication that bacterial growth takes place in ice cream while in storage. In interpreting the results of bacterial examination of ice cream samples due allowance must be made for normal variation in counts, otherwise false interpretations may be made.

The freezing of milk for purposes of transportation and storage has been little used commercially because of the physical effect which makes the remelted product unattractive. However, large amounts of cream are frozen and stored at temperatures below freezing for later use in making ice cream mixes and Webb [168] has recently originated a method for the preservation of whole milk by condensing it and storing the condensed product at —12.2° C. (10° F.). When milk is held at about the freezing point certain types of bacteria grow slowly and materially affect the composition without visible sign of change. In milk held continuously at a temperature of —1.6° C. (29° F.) to 0° C. (32° F.)[116] for 7 to 21 days ice crystals appeared but the milk did not freeze solid. During the period a great increase in bacterial count was observed without visible change in the taste or odor. Other studies [117, 125] have shown similar results. These experiments clearly indicate that milk held at even 0° C. can not be held indefinitely without the growth of bacteria taking place to a greater or less extent, which results in proteolysis of the casein.

In commercial practice it is not customary to hold milk at the freezing point although an attempt is made to approach this temperature. It is obvious from what has been said that cold can not be employed as a means of destroying bacteria in milk, but it is of greatest importance in restraining the growth of microorganisms. Market milk should be held at all times below the minimum temperature for bacterial growth.

The figures in Table XCIV obtained several years ago [114] show as well as any available the effect of temperature over a large part of the growing range of bacteria. The first sample was obtained under good conditions of production, and the second sample, in heavy face in the table, was obtained under ordinary conditions. These figures showing the increase at various temperatures, in relation to time, point out most clearly the effectiveness of decreasing temperatures as they run from the optimum to the minimum.

Effect of cooling milk on the number of bacteria. The use of temperatures below 10° C. (50° F.) is of greatest importance in the production of low count milk. This has been extensively studied by Ayers, Cook and Clemmer [12] with milk produced under different conditions and held at various temperatures: 4.5°, 10°, and 15.5° C. (40°, 50°, and

Table XCIV.—Effect of time and temperature on the growth of bacteria in milk.

Temperature	24 hours	48 hours	96 hours	168 hours
0°C. (32°F.)	2,400 **30,000**	2,100 **27,000**	1,850 **24,000**	1,400 **19,000**
4°C. (39°F.)	2,500 **38,000**	3,600 **56,000**	218,000 **4,300,000**	4,200,000 **38,000,000**
5°C. (41°F.)	2,600 **43,000**	3,600 **210,000**	400,000 **5,760,000**	
6°C. (43°F.)	3,100 **42,000**	12,000 **360,000**	1,480,000 **12,200,000**	
10°C. (50°F.)	11,600 **89,000**	540,000 **1,950,000**		
13°C. (55°F.)	18,800 **187,000**	3,400,000 **38,000,000**		
16°C. (61°F.)	180,000 **900,000**	28,000,000 **168,000,000**		
20°C. (68°F.)	450,000 **4,000,000**	25,000,000,000 **25,000,000,000**		
30°C. (86°F.)	1,400,000,000 **14,000,000,000**			
35°C. (95°F.)	25,000,000,000 **25,000,000,000**			

60° F.), respectively. The milk was examined when fresh and after each interval of 24 hours for 96 hours. The milk was produced under three different sets of conditions as follows:

First, cows were clean and bedded; the udders washed part of the time and left unwashed part of the time; small top pails were used; all utensils were sterilized.

Second, cows were dirty; the manure was removed twice a week; both open and small top pails were used; all utensils were sterilized.

Third, conditions same as second except that utensils were not sterilized.

The results of these experiments are summarized in Table XCV. It will be noted that three different grades of milk were considered, based on their bacterial counts which averaged 4,295, 39,082, and 136,533 bacteria per cc. respectively for the three different conditions. The experiments showed that with two samples of milk of approximately the same initial bacterial count the increase was not always at exactly the same rate.

This difference may be explained by a variation in the bacterial types present, some of which may be favored by the incubation temperatures used. In Table XCV are shown the growth of bacteria in a series of samples produced under the three conditions and having different initial counts when held at different temperatures, and the growth of bacteria in each grade of milk when held at the same temperature. In the lower part of the table are shown the average ratios of bacterial growth arranged to correspond to the counts in the upper portion.

In the sample held at 4.5° C. (40° F.) there was a relatively small growth of bacteria during the period of 96 hours. A most interesting effect of temperature on the growth of bacteria is shown by the samples

Table XCV.—Growth of bacteria in milk held at 4.5°, 10.0°, and 15.5° C. (40°, 50°, and 60° F.)

Average number of bacteria

Temperature	Condition	Fresh	24 hours	48 hours	72 hours	96 hours
°C.						
Section 1						
4.5	1	4,295	4,138	4,566	8,427	19,693
	2	39,082	88,028	121,864	186,245	1,056,922
	3	136,533	281,646	538,775	749,030	852,835
10.0	1	4,295	13,961	127,727	5,725,277	39,490,625
	2	39,082	177,437	831,615	1,761,458	13,079,166
	3	136,533	1,170,546	13,662,115	25,687,541	41,207,272
15.5	1	4,295	1,587,333	33,011,111	326,500,000	962,785,714
	2	39,082	4,461,111	99,120,000	633,375,000	1,355,650,000
	3	136,533	24,673,571	639,884,615	2,407,083,333	5,346,666,666
Section 2						
4.5	1	4,295	4,138	4,566	8,427	19,693
10.0		4,295	13,961	127,727	5,725,277	39,490,625
15.5		4,295	1,587,333	33,011,111	326,500,000	962,785,714
4.5	2	39,082	88,028	121,864	186,245	1,056,922
10.0		39,082	177,437	831,615	1,731,875	13,097,166
15.5		39,082	4,461,111	99,120,000	633,375,000	1,355,650,000
4.5	3	136,533	281,646	538,775	749,030	852,835
10.0		136,533	1,170,546	13,662,115	25,687,541	41,207,272
15.5		136,533	24,673,571	639,884,615	2,407,083,333	5,346,666,666

Ratio of growth

4.5	1	1	1.25	1.21	2.66	5.10
	2	1	2.40	3.80	4.40	22.10
	3	1	2.47	4.62	10.19	11.99
10.0	1	1	3.34	31.07	1,464.70	9,629.60
	2	1	6.70	24.30	86.00	612.30
	3	1	14.68	137.94	498.06	1,385.06
15.5	1	1	398.60	8,772.10	79,809.50	288,231.80
	2	1	351.00	6,114.80	24,990.10	73,920.80
	3	1	759.90	25,308.00	61,527.20	160,873.60
4.5	1	1	1.25	1.21	2.66	5.10
10.0		1	3.34	31.07	1,464.70	9,629.60
15.5		1	398.60	8,772.10	79,809.50	288,231.80
4.5	2	1	2.40	3.80	4.40	22.10
10.0		1	6.70	24.30	86.00	612.30
15.5		1	351.00	6,114.80	24,990.10	73,920.80
4.5	3	1	2.47	4.62	10.19	11.99
10.0		1	14.68	137.94	498.06	1,385.06
15.5		1	759.90	25,308.00	61,527.20	160,873.60

of milk produced under the third condition and held at 10° C. (50° F.). It will be seen that when milk with an average count of 4,295 was held for 72 hours, the average count was but little higher than that of milk with an original count of 136,533 per cc. held 24 hours at the same temperature. A similar condition was found also among samples of low-count milk held 48 hours at 15.5° C. (60° F.), which showed a count of approximately 24,000,000 in 24 hours. At the end of 96 hours the bacterial growth reached a point where the counts were so high as to be approximately the same for all grades of milk.

It is evident that these results have a direct practical bearing, but must be considered in a general way rather than as absolutely quantitative in their application. The figures given in the following discussion must be taken as illustrative rather than absolute.

If milk when fresh averaged approximately 4,000 bacteria per cc., after 48 hours at 10° C. (50° F.) it would contain an average of approximately 127,000. If milk were produced under condition 3, and when fresh averaged approximately 136,000 bacteria per cc., when held for 48 hours at 10° C. (50° F.) its average count would be approximately 13,000,000. The difference in count would be of great importance if an attempt were being made to market a milk of low bacterial count.

The effect of low temperature on the bacterial growth in any one of the grades of milk demands special attention. From section 2 of Table XCV it is evident that even if milk when fresh shows a low bacterial count, the number of bacteria will be high if it is held at a high temperature. For example, milk with an average count of approximately 4,000, when held 24 hours at 4.5° C. (40° F.) showed approximately the same count. At 10° C. (50° F.) the count was about 13,000 while at 15.5° C. (60° F.) the average was about 1,500,000. The results show in every case the great value of holding milk at 10° C. (50° F.) rather than at 15.5° C. (60° F.).

It is realized that night's milk is generally held on the farm for periods of from 12 to 15 hours before delivery; it is, therefore, important to know what bacterial increase will occur in milk held about that period of time. In order to obtain data on the subject, samples of milk produced under clean conditions in sterilized utensils and also samples of milk produced under dirty conditions were held at 15.5° C. (60° F.) and 21.1° C. (70° F.) and examined when fresh, and after 12, 24, and 48 hours. From Table XCVI it will be seen that 16 samples of milk produced under clean conditions in sterilized utensils averaged 3,243 bacteria per cc. when fresh. After 12 hours at 15.5° C. (60° F.) the average count was 4,056 and at 21.1° C. (70° F.) 19,312 bacteria per cc. This shows an advantage of holding at the lower temperature which is more apparent when the milk is held for 24 hours, as the average count was then 123,562 when held at 15.5° C. (60° F.) and 10,006,875 when held at 21.1° C. (70° F.). After 48 hours the average count at both temperatures was high although that of the milk held at 15.5° (60° F.) was the lower of the two.

The samples of milk produced under dirty conditions in unsterilized

Table XCVI.—Growth of bacteria in milk when held at 15.5° C. (60° F.) and 21.1° C. (70° F.).

Milk produced under clean conditions in sterilized utensils.

Sample No.	Fresh	Held at 15.5°C. (60°F.) for— 12 hours	24 hours	48 hours	Held at 21.1°C. (70°F.) for— 12 hours	24 hours	48 hours
1 ..	1,800	2,300	48,000	13,200,000	8,100	4,100,000	1,290,000,000
2 ..	1,900	2,000	56,000	16,500,000	23,100	26,200,000	860,000,000
3 ..	1,700	1,100	55,000	38,000,000	13,600	23,700,000	2,410,000,000
4 ..	3,100	1,800	54,000	....	29,100	12,400,000	....
5 ..	11,200	7,300	83,000	41,000,000	14,700	4,700,000	1,340,000,000
6 ..	7,900	8,700	51,000	57,000,000	37,000	8,200,000	1,810,000,000
7 ..	1,800	2,100	78,000	12,900,000	2,300	510,000	81,000,000
8 ..	700	1,700	28,000	4,800,000	6,800	1,880,000	82,000,000
9 ..	5,600	1,900	23,000	20,100,000	25,200	5,400,000	2,980,000,000
10 ..	1,100	3,100	74,000	42,000,000	19,500	30,600,000	128,000,000
11 ..	3,400	5,100	38,000	14,100,000	13,200	10,200,000	960,000,000
12 ..	1,400	18,300	810,000	....	58,000	5,700,000	....
13 ..	3,100	2,200	60,000	18,100,000	4,100	4,100,000	1,390,000,000
14 ..	1,200	2,000	261,000	46,000,000	32,500	4,920,000	6,400,000,000
15 ..	3,800	3,000	112,000	16,600,000	5,800	6,000,000	6,460,000,000
16 ..	2,200	2,300	146,000	....	16,000	11,500,000	....
Average	3,243	4,056	123,562	26,176,923	19,312	10,006,875	2,014,692,307

Milk produced under dirty conditions in unsterilized utensils.

Sample No.	Fresh	Held at 15.5°C. (60°F.) for— 12 hours	24 hours	48 hours	Held at 21.1°C. (70°F.) for— 12 hours	24 hours	48 hours
1 ..	10,900	15,500	182,000	....	58,000	49,000,000	....
2 ..	1,520,000	8,600,000	148,000,000	169,000,000	12,800,000	460,000,000	1,780,000,000
3 ..	1,880,000	8,700,000	154,000,000	840,000,000	17,600,000	960,000,000	7,500,000,000
4 ..	1,090,000	5,400,000	22,900,000	1,180,000,000	8,200,000	630,000,000	460,000,000
5 ..	2,210,000	11,700,000	81,000,000	28,000,000	13,700,000	149,000,000	620,000,000
6 ..	1,810,000	1,930,000	68,000,000	164,000,000	6,100,000	75,000,000	540,000,000
7 ..	330,000	4,200,000	10,300,000	113,000,000	10,200,000	37,000,000	1,860,000,000
8 ..	159,000	1,210,000	22,600,000	110,000,000	2,830,000	....	350,000,000
9 ..	96,000	1,310,000	29,400,000	....	10,400,000	114,000,000	....
10 ..	37,000	560,000	43,800,000	212,000,000	2,360,000	26,000,000	1,840,000,000
11 ..	18,000	180,000	47,200,000	100,000,000	1,370,000	96,000,000	1,500,000,000
12 ..	12,000	24,000	1,480,000	45,000,000	99,000	32,000,000	2,080,000,000
13 ..	28,000	71,000	2,300,000	....	198,000	35,000,000	....
Average .	707,761	3,376,961	48,550,923	296,100,000	6,608,846	221,916,666	1,853,000,000

utensils ranged from 10,900 to 2,210,000 when fresh and averaged 707,761 bacteria per cc. This high initial average count increased to 3,376,961 after 12 hours at 15.5° C. (60° F.) and to 6,608,846 after 12 hours at 21.1° C. (70° F.). The counts were, of course, very high at later periods at both temperatures. If the ratio of growth of bacteria in Table XCVI is calculated it will be found that there was a higher ratio of growth in milk produced under dirty conditions in unsterilized utensils than in milk produced under clean conditions in sterilized utensils. This seemed to be true when milk was held at 15.5° C. (60° F.) for 12 and 24 hours. At 21.1° C. (70° F.) the statement holds true only for the first 12 hours.

From that and other observations it seems evident that the bacteria which are introduced from unsterilized utensils grow faster at temperatures near 15.5° C. (60° F.) than those in a low count milk produced in sterilized utensils. The results obtained by holding milk at 15.5° C. (60° F.) and 21.1° C. (70° F.) for various periods of time, show the advantage of the lower temperature and further give data on the bacterial increase which will take place at those temperatures when both low and high count milk are held for varying periods of time.

The effect of temperature on the growth of bacteria in milk during storage and transportation is a matter of very great importance. It is evident from the previous results that, if a low count is desired, milk must be cooled and held at 10° C. (50° F.) or lower on the farm, unless it is delivered immediately after each milking. It is important to note that the increase in all samples of milk produced under a given condition is not always the same. This has been observed previously and an explanation given for high counts far above normal. The differences in the rate of growth in milk produced under different conditions of cleanliness are important because they are probably due to different proportions of various groups of bacteria which are introduced into the milk. Not all of these groups will have the same optimum temperature for growth; there will result a difference in the bacterial increase even though the initial contamination is approximately the same in amount.

Another phase of this problem is that of securing low temperatures and the reader is referred to numerous publications on this subject.[24, 65, 66].

The importance of low temperatures in the production and handling of milk deserves most careful study and appreciation by the producer, milk dealer, health official or investigator.

Optimum temperatures. The optimum temperature for the growth of bacteria is of great importance in considering the bacterial flora of milk and the changes produced by growth when the milk has any age, that is, from the time it is drawn from the cow.

Pathogenic bacteria. Pathogenic organisms which are capable of growing in milk have an optimum temperature around 37° C. (98.6° F.) which is about body temperature. Milk, therefore, after it is drawn from the cow is always below the optimum temperature for these types of bacteria. As milk is cooled, the temperature is removed further from the optimum for growth and bacterial development becomes more difficult. *Streptococcus pyogenes* does not grow in milk at 10° C. (50° F.) and it has been found[16] that *Streptococcus mastitidis* does not grow at this temperature. While the last mentioned streptococcus has not been found to be pathogenic to man, it is included with *Streptococcus pyogenes* as an example of how the flora of milk and changes during storage must be viewed at both the optimum and minimum growth temperature.

Experiments by Wahby and Sherman[167] have indicated that, of a number of pathogenic bacteria of milk-borne diseases, all but *Brucella abortus* and *Vibrio cholerae* were able to grow in sterilized milk at temperatures as low as 15° C. (59° F.) and four representatives of the para-

typhoid group grew at 10° C. (50° F.). These facts are advanced as another reason for properly cooling milk.

Peptonizing bacteria. The non-pathogenic bacteria commonly found in milk can be conveniently divided into four groups, peptonizing, inert, alkali-forming, and acid-forming. The peptonizing bacteria as a whole have an optimum temperature around 20° C. (68° F.) to 30° C. (86° F.). In other words their growth in general is most rapid in milk held at room temperatures. Organisms of this group grow also at low temperatures and they are active at points below other groups. Time, however, is an important factor in their growth at temperatures below 10° C. (50° F.) for below this temperature they are also removed from the restraining influence of the development of other bacteria, particularly the acid formers. Considerable attention has been given to the development of the peptonizing bacteria in milk, largely on theoretical grounds. It has been believed that the products of their growth might be toxic if they were unrestrained. This idea has been applied to pasteurized milk in which it was formerly believed that the lactic acid-forming bacteria were destroyed. This was long ago proven erroneous [5] because it was shown that the lactic acid bacteria are not destroyed by pasteurization at the temperature employed. It was further found that the development of the peptonizers in commercial pasteurized and raw market milk was approximately the same.

While the optimum temperature for a general type of microorganism is definitely correlated with growth in pure cultures, other factors come into play when the flora is mixed, as in milk. This is due partly to the reaction of the products of growth of one group or another. In a milk held at 25° C. (77° F.) to 30° C. (86° F.) with both peptonizers and lactic acid-forming bacteria in similar numbers, the peptonizers will be readily overgrown by the acid-forming bacteria because of the restraining action on the peptonizers of the acid formed. When the original numbers of these two groups vary widely the influence of temperature is more important. Data [6] on this point have shown that the development of peptonizers in milk is greatly restrained at a temperature of 10° C. (50° F.) as compared with room temperature.

Among the peptonizing bacteria which have been reported able to grow at low temperatures (10° C. or below) are: *Bact. vulgare, Bact. punctatum, B. mesentericus, B. mycoides, Bact. prodigiosum,*[71] *Bact. granulosum,*[188] and *Bact. proteus, Zopfii* and *Zenkeri.*[85] The "acido-proteolytic" bacteria of Gorini [70] are more proteolytic at lower temperatures. Some of them can resist high temperatures and survive pasteurization and hence may be important in pasteurized milk. Aerobic, spore-forming rods of the subtilis-mesentericus group are among the most actively proteolytic organisms of milk and may be active in the absence of competition from acid-forming bacteria. Members of this group of spore-formers have been found to grow at temperatures of 10° C. (50° F.) or below, others grow at high temperatures and some are truly thermophilic. Organisms of the

group are likely to predominate and cause the spoilage of milk which has been pasteurized at a high temperature.

Inert and alkali-forming bacteria. The inert group of bacteria in milk comprises those which produce no visible change in litmus milk after growth for 14 days at 30° C. (86° F.). This group has not been given much attention largely because of their apparent inertness. Many of the organisms of this group are cocci and their optimum temperature appears to be around 30° C. (86° F.). Alkali-forming bacteria have usually been considered to include all organisms which produce an alkaline reaction and for a long time this alkaline change was attributed to the formation of ammonia. There is, however, a group of organisms [15] which are placed together because they are characterized by their ability to produce an alkaline reaction in milk without visible signs of peptonization. This reaction is due to the conversion of salts of organic acids, present in milk, to carbonates. In litmus milk this alkaline reaction is usually noticeable in 5 days at 30° C. (86° F.) and sometimes it appears in 48 hours. The optimum temperature of this group of alkali-forming bacteria has been found to be between 20° C. (68° F.) and 30° C. (86° F.). Some growth takes place below 20° C. (68° F.) but only slight growth above 30° C. (86° F.).

Some of the inert and alkali-forming bacteria are able to grow at low temperatures. Among these inert bacteria are: *Bact. aquatile,*[138] *Bact. putidum,* and *Bact. stutzeri,*[71] and among the alkali-formers are: *Bact. fluorescens, Bact. alcaligines, Bact. herbicola aureum,*[71] and *Bact. lactis viscosi.*[101]

Acid-forming bacteria. The acid-forming group as applied to the bacteria of milk is a large one comprising as it does micrococci, staphylococci and streptococci from the udder, mouth and feces of the cow; typical and atypical lactic acid bacteria, as *Streptococcus lactis* and variants; the gas-forming bacteria of the colon-aerogenes group; and the high-acid-forming bacteria, as the lactobacilli. Udder organisms, the micrococci, staphylococci and streptococci have an optimum at body temperature, 37° C. (98.6° F.). Of these the streptococci [16] do not grow at 10° C. (50° F.). Streptococci of the feces and mouth of the cow appear to have an optimum temperature of 37° C. (98.6° F.), but grow extremely well at 30° C. (86° F.), while no growth [18] appears to take place at 10° C. (50° F.).

Lactic acid bacteria, which are of vast importance from the standpoint of milk, are represented by *Streptococcus lactis.* This organism has an optimum temperature of 30° C. (86° F.) and as has been pointed out [20, 142] its growth at 10° C. (50° F.) is one of the important characteristics which helps to differentiate it from other streptococci. According to Orla-Jensen,[111] *S. lactis* has a maximum temperature of about 40° C. (104° F.).

Streptococcus kefir,[107, 166] first isolated from kefir, which is characterized by its ability to form gas from sugar,[148] and which is commonly

found in souring milk,[20] grows at about the same temperature as *Streptococcus lactis.*

There are other streptococci found in milk, particularly in pasteurized milk, which are more heat resistant than the typical *Streptococcus lactis,* and which have an optimum temperature around 37° C. (98.6° F.). These bacteria have not been so carefully studied in their physiological reactions but they appear to be closely related to the typical lactic streptococcus and perhaps represent modified strains. From their growth in pasteurized milk held at 10° C. (50° F.) it appears that they have a wide growth range.

Streptococcus thermophilus, one of the lactic acid streptococci usually found in milk and important in making some types of cheese, has an optimum temperature of 40° C. (104° F.) or higher and will grow at temperatures slightly above 50° C. (122° F.). *S. faecium* and *S. glycerinaceus* will grow at the same temperature.[111] According to results published by Orla-Jensen[111] the minimum temperature for growth of *S. thermophilus* is about 20° C. (68° F.), for *S. glycerinaceus* about 10° C. (50° F.) and for *S. faecium* about 6° C. (42.8° F.). In milk held at 45° to 50° C. (113° to 122° F.) these bacteria would usually predominate at first and be followed by high acid bacteria which grow well at these temperatures. In general the lactic acid bacteria commonly found in milk do not grow to any great extent much below 10° C. (50° F.). However, it is important that they do grow *at* this temperature which is the common storage temperature for milk. The restraining action of the development of the lactic group has been mentioned and the effect on the putrefactive types noted. Again it may be well to emphasize the importance of the growth of the lactic acid bacteria in milk at the usual storage temperature. With the slow development of acid the life of the milk becomes more or less automatically taken care of while putrefaction is at the same time retarded.

Gas-forming bacteria. Temperature plays a very important part in the growth and significance of bacteria of the colon-aerogenes group. Their optimum temperature is 37° C. (98.6° F.) and, while they grow readily at room temperature, little growth takes place at 10° C. (50° F.) when milk is held for lengths of time comparable with commercial practice. It has been found[124] that *Escherichia coli* does not grow in milk at 7.2° C. (45° F.) and later studies to determine the significance of the colon count[18] show that organisms of the colon-aerogenes group grow little or not at all in milk held at 10° C. (50° F.) for 24 hours, while in milk held at 21.1° C. (70° F.) for the same period these organisms increase greatly in numbers. Therefore milk storage temperature plays an important part in the growth of these organisms and must be given serious consideration in an interpretation of the colon count. *Aerobacter aerogenes* usually has slightly lower minimum and maximum temperatures for growth than *Escherichia coli.*

High acid-forming bacteria. The lactobacilli include the high acid-forming bacteria of the bulgaricus type. These bacteria do not play any

important part in the flora of fluid milk, but are of great importance in fermented milks and most kinds of cheese. The optimum temperature for most members of the group is about 37° to 40° C. (98.6° to 104° F.), but some members of the group have an optimum temperature above or below these temperatures. Maximum temperatures are as high as 48° or 50° C. (118.4° or 122° F.). The minimum temperature for *L. bulgaricus* (and *L. casei*) is 10° or 15° C. (50° or 59° F.) and for *L. acidophilus* is above 20° C. (68° F.) according to Curran, Rogers, and Whittier.[48] Orla-Jensen[111] found that members of his genus *Thermobacterium* were able to grow at 50° C. (122° F.) or more, but would not grow at temperatures below 22° C. (71.6° F.). This genus includes *Thermobacterium helveticum* (*Lactobacillus helveticum* Bergey),[27] *Tbm. bulgaricum* (*L. bulgaricus*), *Tbm. cereale* (*L. delbrucki*), *Tbm. jugurt* and *Tbm. lactis* (*L. lactis*). His genus *Streptobacterium,* including *Sbm. plantarum* (*L. plantarum*) and *Sbm. casei* (*L. casei*), has a maximum temperature of about 35° to 40° C. (95° to 104° F.) and a minimum temperature of 15° C. (59° F.) or less. *Betabacterium caucasicum,* from kefir, has an optimum temperature below 30° (86° F.) and grows even at 10° C. (50° F.). *Bbm. longum* (*L. longus*) has a maximum temperature of 45° C. (113° F.) and a minimum of about 18° C. (64.4° F.).

Thermophilic bacteria. Of all the groups of bacteria found in milk which have been discussed the maximum temperature for growth is rather close to the optimum while the thermal death point is considerably higher. Generally speaking, the thermal death point of the vegetative cells lies around 60° C. (140° F.) although for some types it may be higher. But there are types of bacteria known as thermophiles, whose optimum temperature is very high and relatively close to their thermal death point. A true thermophile is an organism which *grows* at high temperature and it must not be confused with those bacteria which may endure a high temperature but can grow only at low temperatures. One of these thermophiles which has been found to be one of the causes of "pin-point colonies"[22] has been found to have an optimum temperature between 50° C. (122° F.) and 63° C. (145° F.). This thermophile, termed *Lactobacillus thermophilus,* has a growth range from 30° C. (86° F.) to 65.6° C. (150° F.). It does not grow at 71.1° C. (160° F.) and is killed by 30 minutes' heating at this temperature.

High temperatures—Thermal death point. Above the maximum temperature for growth a point is reached where the bacterial cell is destroyed by heat. This point is known as the thermal death point of the organism, but is not a definite point because the destruction of bacteria is a progressive reaction. The time of heating in relation to temperature plays a very important part. When the thermal death point is discussed, the time factor must always be considered; therefore when a thermal death point is given, the time of heating should also be mentioned.

There are also other factors which influence the thermal death point such as the total acidity and the hydrogen-ion concentration of the medium, the composition and the consistency of the medium, perhaps the

oxidation-reduction potential, and even the gas content. It is generally well known that with the increase in the hydrogen-ion concentration there is a decrease in the thermal death point. The other factors mentioned are less well understood but are suggested as influencing conditions which together with probably many other unknown conditions play a part in determining the thermal death point.

It has been suggested recently [118] that the term "thermal death point" as used by bacteriologists is a misnomer and that the so-called thermal death point is merely a single point selected arbitrarily for descriptive purposes from a continuous series of similar points which taken together make up the time-temperature curve for the species. It is true there has been a tendency to cite figures for thermal death points without due regard for the time factor, but in the application of the thermal death point to industrial processes this factor has been taken into account. It is a mistake to consider the thermal death point as a definite temperature without the time factor. But it can not be assumed that time of holding is the only other factor, as has been mentioned. The thermal death point is as good as any other expression to indicate temperature and time relationship, which with other less well known factors results in the destruction of the bacterial cell.

Bacteriologists have realized for a long time that many factors are involved in the destruction of bacteria by heat, and furthermore, that the destruction is progressive with the rise in temperature as well as the time of holding. Because of this realization the term "majority thermal death point" [66] came into use. This means the temperature in a given time at which the majority of cells are destroyed. This term may be applied to a number of cultures or to the cells of a single culture. To distinguish between the "majority thermal death point" and that point at which after a given time all cells are destroyed the term "absolute thermal death point" is used. It is well to think of the thermal death point in terms of the "majority" and "absolute" points since these points are of importance in studying bacteria which survive the pasteurization of milk.

Space will not permit a detailed discussion of the thermal death points of different milk bacteria as reported by numerous workers. Park [115] has reviewed work on the thermal death points of pathogenic bacteria in milk and has shown that the temperature and time usually used in the holder pasteurization method are sufficient to kill the common pathogens and allow a sufficient margin of safety. Hampil [74] also has reviewed this subject. *Brucella abortus,* especially the porcine strain,[8] has been reported resistant to heat, but most workers claim that it is killed by holder pasteurization.[74, 29] The thermal death points of non-pathogenic bacteria and of bacterial spores are discussed below in a general way. Work on thermal death points of spores has been reviewed by Hampil [74] and Buchanan and Fulmer.[88]

Development of pasteurization. The thermal death point of vegetative cells is relatively low and generally speaking below 60° C. (140° F.) for a period of 10 minutes or more. However, spores of bacteria are

very resistant to heat and may stand boiling for many hours. With a short exposure to heat, temperatures up to 121.1° C. (250° F.) are required. There is also a very marked difference in the effect of moist and dry heat, for much higher temperatures are required when the heat is dry. The destructive effect of high temperatures on bacterial cells, whose thermal death point, in the case of many non-spore-forming bacteria, is not very far above the maximum growth temperature, has led to the application of heat treatment, particularly in the food industry. Pasteur was the first to apply heat in this manner. In studying the diseases of wine from 1860 to 1864, he found that a few minutes' heating at a temperature from 50° C. (122° F.) to 60° C. (140° F.) was sufficient to prevent abnormal fermentations and souring in the wine. A little later he found that by a similar heating beer could be preserved from souring. The application of this process gave rise to the term "pasteurization." This term as applied to milk or cream is the process of heating for short or long periods, as the different processes require, at temperatures ranging between 60° C. (140° F.) and 85° C. (185° F.).

It is claimed that the first commercially pasteurized milk was used in Germany in 1880, and a little later in Denmark. A small home apparatus for heating milk in bottles at boiling temperature devised in Germany [150] did much to attract attention to the application of heat as a means of destroying bacteria in milk and thereby making it a safe food. This method of treatment was suggested for use in this country and as a result the Straus Infant Milk Stations were established.

Methods of pasteurization. There are two general methods for the pasteurization of milk: the first, in which the milk is momentarily heated, is known as the flash process; the second, in which the milk is maintained at the pasteurizing temperature for approximately 30 minutes, is known as the holding process. The flash method is used widely in Europe for market milk and is being used to a greater extent than formerly in this country. It is sometimes used to preheat milk for the holding process and often is employed when the milk is to be used for some purpose other than market milk. In the flash system the milk is usually heated to a temperature around 74° C. (165° F.) for a period of about 30 seconds and, after heating, is rapidly cooled. In a method introduced in Germany, milk spray is rapidly heated in a machine called a "bioriser." Various types of apparatus have been introduced for rapidly heating a continuously flowing, thin layer of milk. Stassano in France has introduced a method called "stassanization" in which the milk passes through the space between concentric tubes and hot water passes through the inner tube and about the outer tube. In the method of Nielsen the milk is circulated in small tubes in a steam chamber. In the Tödt method the milk passes in a thin layer between two casings in the form of truncated cones, and the inner cone rotates. In other methods thin layers of milk are obtained by means of plates. The use of electricity in pasteurizing milk is discussed below. In this process ("electropure") the milk is subjected to an electrical current as it passes between plates.

In the holding process the milk is usually heated to 61° to 63° C. (142° to 145° F.), held at that temperature for 30 minutes and cooled rapidly. This method is the one most widely used in this country and is the only legal method in many municipalities. There are various modifications of the holder method. Usually the milk is held in one tank during pasteurization, but sometimes the milk is merely retarded in its passage through several tanks or tubular passages, so that the milk is supposedly exposed to the pasteurization temperature for the full 30 minutes. Another modification of the holding process is pasteurization of the milk in the bottles. A discussion of the details of the mechanical and operating features of the different pasteurization processes is outside the scope of this book.

Cooling milk after pasteurization. Mention must be made of the necessity for cooling after the process. While sudden changes from a high to a low temperature are of little assistance in destroying bacteria, it is desirable to cool milk quickly and hold it at a low temperature. Pasteurized milk is not sterile milk. It always contains bacteria which will multiply in the milk. For this reason the influence of low temperature in restraining bacterial growth is as important in pasteurized milk as it is in raw milk.

Bacteria surviving pasteurization—General groups which may survive. Early in the development of commercial pasteurization and up to about 1910 there was considerable controversy as to just what happened to pasteurized milk from the standpoint of bacterial growth. In those days it was believed that the thermal death point of the vegetative cells of most bacteria was at 60° C. (140° F.) or below; in other words lower than the usual pasteurizing temperature. From this it could be and was argued that when milk was pasteurized the lactic acid bacteria were destroyed and that the spore-forming peptonizing bacteria which survived were then relieved from the restraining influence of the lactic acid bacteria and when held the milk became putrid rather than sour. It was believed that, through the unhindered growth of the peptonizing bacteria, toxins or other poisonous products were formed which made the pasteurized milk unfit for human consumption.

This view was based, probably, on some work [63] done in Germany in which milk was heated to 70° C. (158° F.) for 30 minutes. The presence of spore-forming peptonizing bacteria which produced highly toxic substances was demonstrated in this milk. This work in 1884 together with the results of studies [62] in this country in 1899 in which it was found that the thermal death point of the lactic acid bacteria ranged from 57° to 60° C. (135° to 140° F.) gave sufficient data for theoretical discussions. From facts of this nature the opponents of pasteurization developed their theory that pasteurized milk when held would become putrid instead of sour and become as a result dangerous for consumption.

There were many, however, who advocated pasteurization as a safeguard against the transmission of infectious diseases and, as a result of this, there was a rather rapid increase in pasteurization in this country

during the period from 1905 to 1910, even though the controversy as to its merit was continuing.

Most of the early work on the bacteria which survived pasteurization was done either on milk heated and held at high temperatures, or upon small samples of milk heated in the laboratory and it was not until 1910 that the results of an extended study [5] of milk pasteurized under commercial conditions appeared. Milk pasteurized in a small city was first studied and this was followed by studies of the development of bacteria in raw and pasteurized milk handled in Boston, New York and Washington. The results brought out some important facts which served as a starting point for the present conception of the type of bacterial growth in commercial pasteurized milk. From the old idea that almost all but spores were destroyed by pasteurization, it would be expected that practically the only types of bacteria left in pasteurized milk would be the peptonizers. Instead, the results showed that the relative proportion of different groups of bacteria in a good grade of pasteurized milk was approximately the same as that in a good grade of clean raw milk. The alkali or inert group formed in both cases the majority of the bacteria and, as the milk was held at 10° C. (50° F.), it increased in its proportion during the first day, then gradually decreased until the fifth day, when its proportion was less than the lactic acid group. The acid bacteria constituted, on the initial count, from 20 to 30 per cent of the total and after a decrease in proportion during the first 24 hours they gradually increased to form the largest portion of the bacterial content of the sixth day. The peptonizers were the smallest group in both raw and pasteurized milk. They increased slightly in proportion and then decreased.

When the same grades of milk were held at room temperature the groups stayed in the same relative proportions, but the lactic acid group reached the ascendancy at the end of 48 hours instead of on the sixth day. The peptonizing group was again low in proportion and after a slight increase, a decrease occurred.

In obtaining these results litmus-lactose agar and gelatin plates were used for the differential count and, as was found by the authors in later studies, the lactic acid group probably comprised only the more active types when differentiated by this method. Some of the weak and slow acid-formers were undoubtedly included in the alkali or inert group; so this group is in reality not as large as it appears from these results.

The following conclusions bring out some of the results of the study of the bacteria in raw and commercially pasteurized milk, which included milk pasteurized both by the flash and by the holding processes:

1. "Commercially pasteurized milk always sours, because of the development of lactic acid bacteria, which, on account of their high thermal death point, survive pasteurization, and perhaps in some cases because of subsequent infection with acid-forming bacteria during cooling and bottling. The acid development in an efficiently pasteurized milk is about the same as in a clean raw milk. But sometimes a strong old taste develops which is probably due to the development of alkali or inert

bacteria. The old taste is not characteristic of pasteurized milk, for it may be noticed in clean raw milk when held under similar conditions. The less efficient the pasteurization process, the more closely does the acid increase of the heated milk approach that of a dirty raw milk.

2. "The relative proportion of the groups of peptonizing, lactic acid, and alkali or inert bacteria is approximately the same in efficiently pasteurized milk as it is in clean raw milk. In both cases the alkali or inert bacteria form the largest group, the lactic group is next, while the peptonizers are in the minority. At the time of souring the group proportions have changed so that the lactic acid bacteria constitute the largest group, with the alkali or inert next in order and the peptonizers the smallest portion as initially. In both of these milks the group of peptonizers may increase slightly in its proportion to the other two groups during the first two days, but it then gradually decreases and always forms the smallest group.

"When milk is less efficiently pasteurized the position of the groups may be reversed so that the lactic acid bacteria constitute the largest group with the alkali or inert forms next in order, but here again the peptonizers form the smallest proportion to the total bacteria. This group arrangement is the same as in a dirty raw milk. The more efficient the pasteurization the smaller the percentage of lactic acid bacteria and similarly, the cleaner the raw milk the smaller the percentage of lactic acid bacteria."

These results show very clearly that the old ideas of the putrefaction of pasteurized milk and lack of souring, as it is commercially treated and handled, do not hold. The fact is that the thermal death point of the vegetative cells of many bacteria is sufficiently high to permit them to survive the low temperatures now universally employed in commercial pasteurization. The fact that some strains of lactic acid bacteria are not destroyed by pasteurization is very important since they may play an important rôle in restraining the development of the peptonizing types of bacteria.

If a very low count milk is heated to 63° C. (145° F.) for 30 minutes in a flask and then allowed to stand at room temperature, a sweet rennet curd may form and putrefaction follow with little or no acid development. This may be due to a lack of heat resistant lactic types in the low count milk or the fact that the temperature is a little high. It may also be due to the fact that the milk is not cooled after pasteurization. In commercial practice milk is always cooled after pasteurization, even though later on it may be removed from the ice and held at room temperature in the home. Nevertheless, the influence of the cooling followed by cold storage for 8 to 12 hours is usually important. It has been observed many times that this cooling restrains the growth of the peptonizing forms and that during this period the acid formers have time to start growth so that later when held at room temperature their rate of growth hinders the peptonizers.

In a study of the bacteria which survive pasteurization, the influence

of storage temperature has been carefully observed under laboratory conditions and the following conclusions were reached.

"When different grades of milk are pasteurized at 63° C. (145° F.) in a laboratory and held at room temperature the bacterial flora may undergo three distinct changes. First, when a fair quality milk is pasteurized, the acid group may develop at once and overgrow all the other groups, forming acid and producing an acid curd. Second, when a poor quality milk is pasteurized the peptonizing group may grow rapidly at first along with the acid group which later overgrows it. In this case, the milk will become curdled with a rennet curd due to the peptonizing bacteria, then later will become sour from the development of the lactic acid group of organisms. Third, when a good grade of milk is pasteurized the peptonizing bacteria may overgrow the acid group of organisms so that the milk becomes peptonized without development of any acid. The same grades of milk treated in the same manner but held in an ice chest at 10° C. (50° F.) show entirely different changes in their bacterial contents. The growth of the peptonizing group is restrained so that they are of little importance. The percentage of the acid group remains the same through a long period. Occasionally the percentage of the alkali group may increase after five days, but eventually the acid group forms the major group. These results were obtained from laboratory experiments and only indicate the possible changes in the bacterial flora of pasteurized milk when held at different temperatures. They show the delicate balance between the bacterial groups, but can not be applied to indicate the bacterial changes in milk pasteurized and handled under commercial conditions."

Ability of streptococci to survive pasteurization. It has been frequently observed that there are resistant lactic acid bacteria. In pasteurizing milk by the flash process at 85° C. (185° F.) it was found [129] that resistant types occasionally survived the process. Living Güntheri organisms were also found by Wolff [176] in milk pasteurized at 70° C. (158° F.) for one-half hour, although he [175] and Weigmann [170] found that the lactic acid bacteria are greatly weakened by the heat of pasteurization in growth as well as in acid production. In a study [69] of milk pasteurized in bottles for 30 minutes at 65° C. (149° F.) it was found that the bacteria which resisted belonged for the most part to the inoffensive lactic organisms.

Studies made [5] on this subject using 45 cultures of lactic acid bacteria isolated from milk showed that the thermal death point when heated in milk in Sternberg bulbs for 30 minutes was 75.6° C. (168° F.). It is this heat resistant type of lactic acid bacteria which causes the souring of commercially pasteurized milk and it is commonly found in raw milk. Of the lactic acid bacteria in raw milk the average percentage which survive pasteurization for 30 minutes at 63° C. (145° F.) has been found [6] to range from 1.27 to 4.55 per cent in various grades of milk.

Studies of the ability of streptococci to survive pasteurization [8] revealed facts which are of significance in connection with the bacteriology

of pasteurized milk. The thermal death points of 139 cultures of streptococci isolated from cow feces, the udder and mouth of cows, and from milk and cream showed a wide variation when heated in milk for a period of 30 minutes. About 33 per cent of the streptococci studied survived heating at 63° C. (145° F.) for 30 minutes. As this is the usual pasteurizing temperature and holding period, this fact is significant. Of even greater interest is the fact that streptococci from different sources showed a difference in heat resistance. The streptococci from the udder on the whole were less heat resistant and those from milk and cream more resistant than those from the mouth and feces of cows. Of 180 cocci isolated by Hucker [87] from pasteurized milk *Streptococcus thermophilus* Orla-Jensen was the predominating organism. In a recent study [147] of a group of high-temperature streptococci isolated from milk and other sources, all survived heating at 30 minutes at 62.8° C. (145° F.). This group included *Streptococcus thermophilus, S. bovis, S. inulinaceus, S. fecalis, S. glycerinaceus, S. liquefaciens,* and *S. zymogenes.*

Studies of the streptococci [8] which survive pasteurization showed that in general there are two classes of streptococci: Class 1, those streptococci which may have a low "majority thermal death point" but a high "absolute thermal death point"; and Class 2, those streptococci which have a high "majority thermal death point."

In Class 1, the "majority thermal death point" might be below the pasteurizing temperature and most of the bacterial cells would be destroyed. However, a few cells, more resistant than the others, might survive the heating and continue to grow. The experimental results plainly showed this to be the case with some cultures of streptococci, for often in a tube of milk containing a culture of streptococci which had been heated the reaction indicating growth was shown in 24 hours; in other cases five or six days' incubation was necessary to show the reaction. When the reaction was delayed it was evident that the heating destroyed most of the cells and that those few cells surviving required a longer incubation in order to multiply to a point where the typical reaction indicating growth was visible.

The second class of streptococci survive pasteurization because their "majority thermal death point" is above the pasteurizing temperature. Ayers and Johnson [8] found that when milk was heavily inoculated with a lactic acid-forming streptococcus known to have a high thermal death point and samples of the milk were then pasteurized at three different temperatures for 30 minute periods, there was no destruction of the streptococci at 60° C. (140° F.) or at 65.5° C. (150° F.) but at 71.1° C. (170° F.) 99.2 per cent of the streptococci were destroyed. Streptococci having this high majority thermal death point survive commercial pasteurization at 63° C. (145° F.) for 30 minutes because their thermal death point is above this pasteurizing temperature.

These streptococci with a high majority thermal death point are extremely important because they are responsible for the natural souring of commercially pasteurized milk. However, the presence of large numbers

in raw milk frequently disturbs the milk dealer and health officials because of the resulting high bacterial counts. Frequently daily bacterial counts of pasteurized milk will run uniformly low for weeks at a time. Then suddenly the count will increase and remain high; then, just as suddenly drop to a low point again. This sort of thing is readily understood if the possible presence of heat-resistant streptococci is kept in mind. If, for any reason, the number of these organisms increases above the average in raw milk, the numbers after pasteurization will be correspondingly high because these organisms with a high "majority thermal death point" will pass through the process without being affected by the heating. If these organisms are present in considerable numbers in pasteurized milk their colonies on plates at the usual low dilution of 1 to 100 will be very small on account of overcrowding as well as because of poor growth on the standard agar medium. Under these conditions the colonies may appear as pin point in size. The appearance of pin-point colonies, which has disturbed those interested in the bacteriology of milk, is due in part to these types of heat-resistant streptococci. Studies of pin-point colonies [157] observed in milk in Baltimore indicated heat-resistant streptococci as the cause. Of 52 cultures examined 50 were found to be streptococci. Other examinations of the cause of abnormally high plate counts on pasteurized milk when pin-point colonies were present have revealed streptococci.[93] The organisms were found not to be destroyed at a temperature of 63° C. (145° F.). In a more recent study [120] streptococci were found to make up a large proportion of the heat-resistant and thermophilic bacteria which survived pasteurization at 62.8° C. (145° F.) for 30 minutes.

The appearance of increased numbers of these heat-resistant streptococci seems to occur most frequently in raw milk in the spring and summer months. They can generally be traced to one or more shippers and, if the proportion of this milk is sufficiently high to raise the final count of the pasteurized milk from a given plant, the trouble may be eliminated by rejecting the raw milk from the farm. Sometimes this is done and steps are taken to sterilize all equipment at the farm and improve the conditions of production until the milk becomes normal in count of heat-resistant streptococci.

Since the streptococci represent a large variety of bacteria and include harmless lactic types as well as extremely virulent hemolytic types it is natural to wonder if pasteurization destroys this latter pathogenic type.

All experimental evidence as well as the results of practical experience shows that the thermal death point of pathogenic streptococci is sufficiently low so that they are destroyed by proper pasteurization at 63° C. (145° F.) for 30 minutes. Twenty-four strains of pathogenic hemolytic streptococci were found to be destroyed by heating to 60° C. (140° F.) for 30 minutes.[49] Later studies [14] with 27 strains of pathogenic streptococci from pathologic sources showed that the thermal death point when heated in milk for 30 minutes ranged from 52° to 60° C. (125° F. to 140° F.). Of these 5 were destroyed at 52° C. (125° F.), 5 at 54.5° C. (130° F.), 12 at 57° C. (135° F.) and the remaining 5 of the 27 strains

at 60° C. (140° F.). These figures clearly indicate that there is no cause for suspecting the survival of pathogenic streptococci in the process of pasteurization, if the milk is pasteurized at 63° C. (145° F.). In commercial practice an ample margin of safety must be allowed because of the variation in temperature which occurs when a large volume of milk is heated.

Ability of colon bacilli to survive pasteurization. The question whether colon bacilli—the term in this case including bacteria of the colon-aerogenes group—can survive pasteurization is of interest because of the possibility of using the colon test as an index of efficiency of pasteurization.

In 1899 it was found [168] that *Bacillus neapolitanus* of Emmerich—the same organism as *B. coli communis* of Escherich—was destroyed by heating for 5 minutes at 59° C. (138° F.) and one minute at 62.5° C. (144.5° F.). On the basis of these results a study [127] was made of pasteurized milk from 24 dairies in Amsterdam. As colon bacilli were found in pasteurized milk from 10 of the 24 dairies it was concluded that this proportion, or 41 per cent, did not pasteurize or handle the milk properly.

Numerous investigators have studied cultures of *E. coli* and found that the organisms were easily destroyed at temperatures below 62.8° C. (145° F.). At times, however, it has been found that high temperatures were required to destroy this organism and heat-resistant strains have been observed.[25, 51, 66, 94, 149, 159, 180] In a study [9] on the ability of these organisms to survive pasteurization it was found that certain strains could survive heating at 63° C. (145° F.) for 30 minutes. On examination of 174 cultures of colon bacilli, it was found that at 60° C. (140° F.), the lowest pasteurizing temperature, 95 cultures survived; at 63° C. (145° F.), the usual temperature for pasteurization, 12 survived. In each case the heating period was 30 minutes. Considerable variation was observed in the thermal death point of the colon bacilli which survived at 63° C. (145° F.). When the cultures which withstood the first heating were again heated it was found that many did not survive, and in each subsequent heating different results were obtained. Colon bacilli have a low "majority thermal death point" but, on account of the resistance of a few cells, they may survive the pasteurization process.

The colon test as an index of the efficiency of the process of pasteurization is complicated by the ability of certain strains to survive a temperature of 63° C. (145° F.) for 30 minutes and to develop rapidly when the pasteurized milk is held under certain temperature conditions met during storage and delivery. Consequently the presence of a few colon bacilli in pasteurized milk under ordinary market conditions does not necessarily indicate that the milk was not properly heated. The presence of a large number of colon bacilli immediately after the heating process indicates that the milk has not been heated to 63° C. (145° F.) for 30 minutes, and this test properly applied should be valuable in control work. Many investigators [86, 92, 104, 158] believe that the colon test has a place in

pasteurization control as a supplementary test of quality and Savage[187] suggests that an appropriate standard would serve as a useful check on excessive recontamination of milk. As already pointed out,[148] however, the colon test as now used and advocated in some localities, is misleading, particularly when used for milks in which bacterial growth has taken place. Under such conditions this test is of little value as an index to the sanitary conditions surrounding the production of raw milk of the usual market grade. As indicated, the test may have application in high grade milks as a supplementary test of quality.

Fermentation tubes can be used for making the test, but when gas formation is noted the presence of colon bacilli should be demonstrated by further tests. Often anaerobic spore-formers are encountered which survive pasteurization and give the typical fermentation tube test. It has been shown[6] that when fermentation tubes with lactose bile or dextrose liver broth are used in the search for organisms of the colon-aerogenes group in pasteurized milk, gas formation may be observed which is not due to this type of organism. This gas formation was found to be due to a spore-forming anaerobic bacillus of the butyric group. For this reason, if fermentation tubes are used in a presumptive test for the presence of organisms of the colon-aerogenes group, the actual presence of numbers of this group must be confirmed by other suitable tests.

Ability of alkali-forming and peptonizing bacteria to survive pasteurization. The alkali-forming bacteria are quite likely to survive the pasteurizing process because their thermal death point ranges from 60° to 65° C. (140° to 150° F.). These bacteria are characterized by their ability to produce an alkaline reaction in milk without visible signs of peptonization. As has been previously stated in this chapter this alkaline reaction is due to conversion of salts of organic acids to carbonates. Alkali-forming bacteria include both cocci and bacilli and are an extremely interesting group. Those which survive pasteurization have been rather extensively studied[15] and given an arbitrary grouping. Black, Prouty, and Graham[28] studied the effect of pasteurization on the bacterial flora of low count milk. In the raw milk the alkali-forming and inert bacteria composed 73.07 per cent of the bacterial flora present. In the freshly pasteurized milk the alkali-forming and inert group composed 84.74 per cent of the total. The proportion of the acid-producing and proteolytic organisms declined as a result of pasteurization. Alkali-forming bacteria grow in pasteurized milk but slowly, if at all, at the temperature of the ice box. Theoretically, they might retard souring by the neutralization of the acidity but practically it is doubtful if the alkali formation is sufficiently great to play any observable part in the reaction.

The peptonizing group of bacteria which survive pasteurization are of importance since they are the bacteria originally believed to make pasteurized milk dangerous. Their growth in average raw and commercially pasteurized milk has been shown, however, to be about the same in corresponding grades of milk. In low count raw milk,[28] however, the proteolytics increase at 7.1° C. (45° F.) but decrease at 19.9° C. (68° F.)

while in pasteurized milk they increase considerably at both temperatures. Under the usual conditions the peptonizers are readily overgrown and while their numbers increase in milk their proportion to the total constantly decreases. Contrary to what might be expected, it has been found that of a total of 50 cultures of peptonizing bacteria isolated from pasteurized milk, 3 were spore-formers; this is equivalent to 6 per cent. The same investigators also found that of 225 cultures of groups isolated from many samples of pasteurized milk, 3, or 1.35 per cent were spore-formers.

Hypothetical grouping of bacteria in raw and in pasteurized milk. From studies of bacteria which survive pasteurization the hypothetical relation of the bacterial groups in average raw milk and in milk pasteurized by the 30-minute holding process at various temperatures has been worked out from studies under laboratory conditions.[6] The bacterial flora of various kinds of milk is represented in Figure 37 by columns of equal length divided in sections which in a general way show the relative proportions of the bacterial groups. The proportions were worked out from the milk tube method of differentiation which is the reaction observed in litmus milk tubes after 14 days' incubation at 30° C. (86° F.). The method of procedure is to pick each colony on the plate, inoculate into litmus milk, and then to group the bacteria on the reaction produced. It is more accurate than the litmus-lactose gelatine plate method and shows more acid-forming bacteria. From Figure 37 it appears that raw milk contains four principal groups of bacteria,—the acid, inert, alkali, and peptonizing. The acid group is divided into two sub-groups, the acid-coagulating which coagulates milk in less than 14 days, and the acid group which merely produces acid. In raw milk the inert group is the largest. In milk pasteurized at 63° C. (145° F.) the great increase in the proportion of the acid-coagulating and acid groups may be noticed while the percentage of the peptonizing group is reduced. In the study [28] in which low count raw milk was used pasteurization reduced the proportion of both the peptonizers and acid-forming group. At 71° C. (160° F.) the total acid group is still the largest but the bacteria do not seem to grow and form acid as quickly or to the same degree as those which make up the group which survives the lower temperature. At this temperature the alkali and peptonizing groups are reduced to a minimum. At 77° C. (170° F.) the total acid groups remain about the same but the organisms produce acid slowly. The alkali group is practically destroyed. The most important change is in the peptonizing group which at this temperature begins to increase in proportion. This increase is more striking at 82° C. (180° F.) where 75 per cent of the bacteria which survive are peptonizers. About the same relations hold at higher temperatures. It must be remembered that these relations were worked out on averages of a large number of samples of milk and the bacterial flora of every sample of milk must not be expected to conform exactly to these averages.

Survival and growth of bacteria in foam. The foam which forms on the surface of the milk in the vats of the holding process and the milk which splashes on the top and sides do not ordinarily reach the tem-

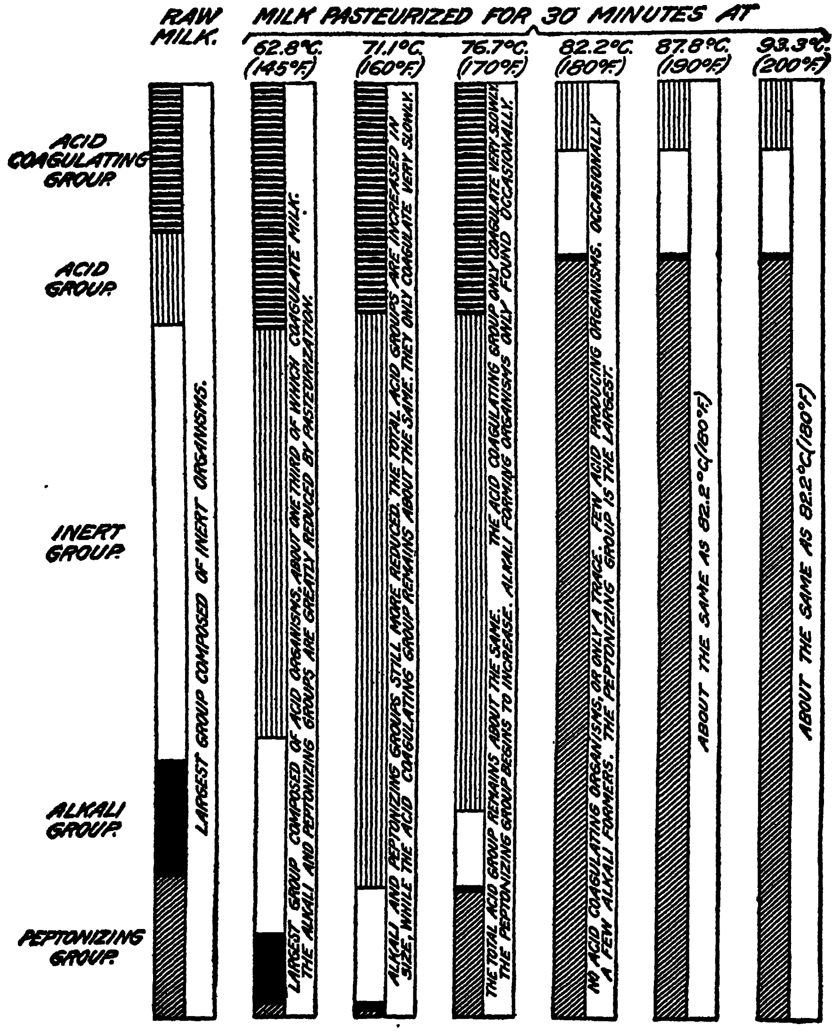

FIG. 37.—The hypothetical relation of bacterial groups to raw and pasteurized milk.

perature of the milk below. Consequently there may be a survival of a number of bacteria, including pathogens, which could not survive the temperature of the milk; and there may even be an increase in the numbers of some organisms.

Siebel[141] has studied the ability of a number of common milk organisms to resist pasteurization at 63° C. (145° F.) for one-half hour and said that most of them would be able to survive in the foam. He stated that the temperature of the foam was considerably lower than that of the milk but gave no figures. Whittaker, Archibald, Leete and Miller[172] found that the average temperature of the foam was 3° C. (5° F.), or

more, lower than the temperature of the corresponding milk. They reported that the number of bacteria in the foam was always considerably higher than the number in the corresponding pasteurized milk and that an increase in numbers of bacteria in the foam had taken place in two-thirds of their samples. The foam never completely leaves the vat during a day's run and serves as a medium for the growth of some bacteria and as an inoculum for the milk as it leaves the vat.

Attempts have been made to heat the foam layer up to the temperature of the milk. This is usually accomplished by hot air or steam which is introduced into the air space above the foam. In many of the high-temperature short-time pasteurizers the milk is wholly or in part under pressure and therefore enclosed which automatically eliminates foam and "splash."

Effect of pasteurization on number of bacteria. With the increase in the use of the bacterial count in connection with the control of the milk supply there has been an increasing desire to obtain low counts in pasteurized milk. In the early days of pasteurization it was customary to determine the efficiency of the process by the per cent reduction. A reduction of at least 99 per cent was expected. As more was learned regarding the heat resistance of bacteria and their variable appearance in raw milk the absurdity of measuring the efficiency of pasteurization by percentage reduction became apparent.

As a general rule the higher the count of the raw milk the greater is the percentage reduction through pasteurization. Ayers and Johnson [6] have shown that when raw milk containing over one million bacteria per cc. was heated at 63° C. (145° F.) for 30 minutes in a flask, only 3 out of 28 samples showed less than 99 per cent reduction. However, 13 of the samples of pasteurized milk contained over twenty thousand bacteria per cc. Similar experiments in which the raw milk contained less than one million bacteria per cc. showed quite different results as 14 out of 24 samples showed a percentage bacterial reduction of less than 99 per cent. One sample of milk before heating contained 119,000 bacteria and after pasteurization 20,800 per cc., a percentage reduction of only 82.52 per cent. This sample evidently contained a high percentage of heat-resistant bacteria which survived the process of pasteurization. In milk containing about 3500 bacteria per cc., Black, Prouty, and Graham [28] obtained a percentage reduction of only 90 per cent. In this connection it has been shown [146] that the age of the bacterial cells influences greatly the susceptibility of the organisms to heat, young cells being more easily killed than older ones. Consequently a higher percentage destruction of bacteria by pasteurization may be expected in milks which have been held at temperatures which allow the multiplication of the bacteria contained therein than in milks of the same original flora which have been stored at temperatures sufficiently low to inhibit bacterial growth. These results make it evident that percentage bacterial reduction is no measure of the efficiency of pasteurization. It is evident that bacterial standards for pasteurized milk must not be set below the limit which it is possible to obtain.

Rate of bacterial growth in pasteurized milk. The assertion has been frequently made that bacteria grow faster in pasteurized milk than in raw milk. At times the mistake has been made of comparing growth in raw and pasteurized milk when the bacterial count at the beginning was quite different. In order to make a fair comparison of the rate of growth in raw and pasteurized milk, samples of milk having about the same number of bacteria must be selected. Ayers [5] studied this subject and concluded that the rate of growth in pasteurized milk is not greater than in raw milk when milks of a similar bacteriological quality are compared. Somewhat later Allen [1] reported that raw milk as compared with pasteurized milk exerts a powerful restraining influence upon the multiplication of *B. lactis acidi* and a chromogenic bacterium. Bogdanoff [80] observed that as the temperature was increased above 55° C. (131° F.) or 60° C. (140° F.) the milk became more favorable to the growth of *Streptococcus lactis* and *Lactobacillus casei.* The effect of pasteurization of milk upon its growth-promoting properties has been variously attributed to changes in the colloidal dispersion system [80] of the milk, its destructive action upon leucocytes,[171] or germicidal activity, inactivation of inhibiting heat-labile substances, or improvement in the nutritive properties of the milk.

Interpretation of bacterial counts of pasteurized milk. In commercial pasteurization the important things to be controlled are the temperature, time and method of heating and the prevention of reinfection. The bacterial count assists in such control when properly used and interpreted.

The bacterial count of pasteurized milk is more complicated than that of raw milk when it comes to interpretation, and bears only a general relation to the count before pasteurization. The interpretation of a bacterial count of any milk, whether raw or pasteurized, can at best serve only as a general index and should be used with this idea in mind. When made under definite conditions with the same medium and the same methods, counts provide a picture of a combination of conditions with regard to milk which, if interpreted correctly, give an idea of any important changes in these conditions. Such information provided by routine tests should serve to point out unusual changes which require further investigation. When used in this manner, routine bacterial counts made by the laboratories of health departments and milk dealers are valuable.

In order to interpret the count properly, consideration must be first given to where and when the sample was taken. If the sample represents freshly pasteurized milk, and is taken at the plant immediately after bottling, the count measures the numbers of bacteria which survive the heating process, together with any contamination through machinery and bottles. If taken from delivery wagons, the count will measure the same thing, but also, in addition, any growth which may have taken place. When the counts are high in fresh bottled pasteurized milk, they may be due to a contamination, to high-count raw milk, to an unusually high proportion of resistant types, or, in special cases, to growth of a thermophilic organism which will be discussed later.

In order to determine which of these causes is responsible, further investigation is required in which a series of samples are taken in each step of the process. The interpretation of the results of such a series is again complicated, because of the fact that there is no assurance that the samples represent the same milk at each step in the process. In some plants, it is, of course, possible to take samples in such a way that some of the milk can be examined at various stages during pasteurization. In other plants there is a continuous flow of milk, so varying in kinds and numbers of bacteria as to complicate seriously proper sampling. These points are emphasized so that there may be no false idea prevalent that series samples on test runs can be analyzed and interpreted in a haphazard manner. It must be borne in mind that it is impossible to interpret properly bacterial counts of pasteurized milk or the results of special tests without a fundamental understanding of the bacteria which survive the process.

Importance of "majority" and "absolute" thermal death points. The "majority" and "absolute" thermal death points of bacteria are sufficiently important from the standpoint of pasteurization to warrant further consideration. Some interesting experiments [21] have been conducted which showed that the "majority" thermal death point of an *Aerobacter aerogenes* culture studied was below 57° C. (135° F.) and the "absolute" thermal death point a little above 65.6° C. (150° F.) when the temperature was maintained for a period of 30 minutes in milk. When 100 cc. of infected milk was heated at 57° C. (135° F.) surviving cells could be detected by examining 1 cc., or in other words, by examination of 1 per cent of the total volume of milk studied. When heated to 60° C. (140° F.) it was necessary to examine 2 per cent of the milk to find surviving cells. At 63° C. (145° F.) for 30 minutes no living organism could be detected when 1 per cent (1 cc.) was examined nor when in addition 14 per cent of the total volume of milk was examined, but there were surviving cells in the remaining 85 per cent of the total volume.

These results were interpreted in terms of large scale efficiency tests. Suppose a test is being made in which instead of 100 cc. of milk infected with *A. aerogenes* there had been 1,000 pounds. Calculating the results on this basis when heating to 60° C. (140° F.) 10 pounds of milk, approximately 4,800 cc. or 1 per cent of the total volume could have been examined without detecting the survival of living cells, although they could have been found by the examination of 2 per cent of the total volume, this being 20 pounds or about 9,000 cc. In a test therefore with 1,000 pounds of milk the examination of 4,500 cc. of heated milk would, on the basis of the results presented, have shown no living cells of the test culture, and a temperature of 60° C. (140° F.) for 30 minutes would therefore be considered effective for destroying all cells. The seriousness of the interpretation of results in this manner is at once evident when it is remembered that similar differences between the "majority" and "absolute" thermal death points probably exist among the pathogenic bacteria.

In a similar manner the results obtained by heating the *A. aerogenes* culture to 63° C. (145° F.) for 30 minutes may be calculated on the basis of a larger experiment using 1,000 pounds of milk. It will be remembered that no living cells were found when 15 per cent of the total volume of heated infected milk was examined but were present in the remaining 85 per cent.

The conclusions to be drawn from these experiments are of importance. In large scale efficiency tests of pasteurizers the amount of milk which can be subjected to examination is naturally limited and the results if positive,—that is, if the test organisms survive,—show that the temperature is too low. If the results are negative,—that is, show no evidence of living cells of the test culture,—then the only safe interpretation is that there are none present in the amount of milk examined. Nothing can be said of the rest of the milk in the test. It is because of the wide range between the "majority" and "absolute" thermal death points of bacteria that this situation arises.

In view of these facts it is obvious that the selection of an effective pasteurizing temperature must be based, as it has been, on the "absolute" thermal death point of pathogenic organisms determined under laboratory conditions. Large scale efficiency tests are unnecessary and are likely to be misleading. Knowing the "absolute" thermal death points of pathogenic organisms the most valuable work for the future, in connection with pasteurization, will be a study of temperatures obtained in a commercial practice and the development of suitable instruments for determining that all the milk in a given pasteurizing process is heated to a point which will provide a safe margin above the "absolute" thermal death point of pathogenic organisms.

Based on the extensive studies of the thermal death points of pathogenic bacteria which have been transmitted through milk, a chart has been prepared [110] showing the temperature and time curve necessary for their destruction, together with the chemical effect and effect on the cream line. This chart, Figure 38, gives a picture which is valuable but must not be too strictly interpreted.

Thermophilic bacteria—Pin-point colonies. Bacteria of an interesting type have been found in the soil and in hot springs. They grow at high temperatures destructive to most vegetative cells. Such organisms are known as thermophiles. True thermophiles not only survive high temperatures around 63° C. (145° F.) but reproduce between 55° C. (131° F.) and 75° C. (167° F.).[128] They must not be confused with those organisms which resist this temperature but do not grow. Heat-resistant bacteria of this kind have been described above.

Until rather recently but little attention was given to the growth of thermophiles in milk. As early, however, as 1867 [109] it was observed that when milk was held at 50° C. (122° F.) the milk became sour. This was also observed later [102] and it was pointed out that the fermentation was not due to ordinary milk-souring organisms. In an investigation as to the possibility of transporting milk in a hot condition it was found [17] that

the growth of thermophilic bacteria complicated the problem. From time to time bacterial counts on pasteurized milk have suggested the growth of thermophiles, and in one experiment [75] it was found that the count in pasteurized milk held from 4 to 6½ hours at the pasteurization temperature was higher in most cases than when the milk was held 30 minutes.

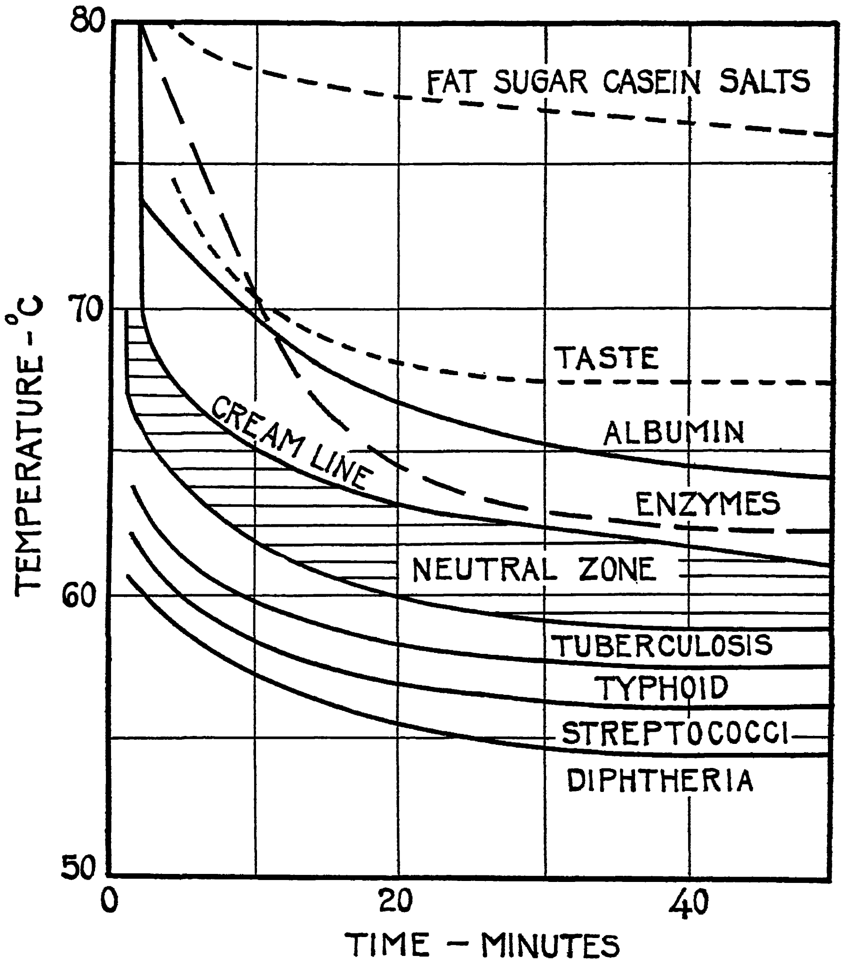

FIG. 38.—Effect of heating on the constituents of milk and on certain pathogenic bacteria. (From North.[110])

Lactobacillus thermophilus and other thermophiles. A thermophilic streptococcus called *Streptococcus thermophilus* was described by Orla-Jensen [112] a number of years ago. A true thermophilic organism has been isolated from pasteurized milk by Ayers and Johnson and shown to be one of the causes for pin-point colonies. This organism was described by these workers and termed *Lactobacillus thermophilus.* Robert-

son[128] later described the same, or a closely related bacterium, and *Streptococcus thermophilus,* both of which were isolated from pasteurized milk and formed pin-point colonies. The latter organism is one of the most frequently encountered in pasteurized milk, particularly in milk which has been held for some time at 30° C. (86° F.) prior to pasteurization. While it will grow at 65.6° C. (150° F.) it is killed by heating at 71° C. (160° F.) for 30 minutes and at 82° C. (180° F.) for two and one-half minutes. The close relation between the maximum growing temperature and the "absolute" thermal death point is of interest. The source of these organisms is not definitely known. Sherman and Stark[147] have extended our knowledge of the high temperature streptococci found in milk.

The significance of pin-point colonies due to Lactobacillus thermophilus. Since this organism is present in raw milk in small numbers, and will develop rapidly at the pasteurizing temperature, it is evident that a cumulative infection of tanks and other dairy machinery may take place unless the cleaning and sterilizing process is performed each day in such a manner as to destroy the organism. If the pasteurizing tanks are properly cleaned and are in perfect condition, without leaks into the insulating walls, and are thoroughly steamed and all parts heated to at least 82.2° C. (180° F.), there should be little trouble from this thermophile. Steaming of the cooler and bottler should of course be included. This sterilizing process must be in daily operation, preferably just after the pasteurizing run. It may even be that the pasteurizing run will have to be stopped and the tanks given a steaming during the daily operation.

From a sanitary standpoint, except as an indication of improper sterilization of equipment which may result in high counts, the presence of the organisms appears to be unimportant. They have been consumed for a considerable period of time with no indication of harmful results. *Lactobacillus thermophilus* appears to be a harmless saprophytic nonpathogenic thermophile, which is probably always present in raw and pasteurized milk in variable numbers.

Causes of pin-point colonies. Before taking up the general causes of pin-point colonies it must be realized that colonies may become pin point in size if there are large numbers of them on a plate. Colonies are called pin points when they are just visible to the naked eye and appear very small under a hand lens. Suppose that a sample of milk is plated using a dilution of 1 to 100. If the milk contains 15,000 per cc. then there will be 150 colonies on the plate and the colonies will appear in their normal size. On the other hand if the milk contains 150,000 bacteria per cc. then there will be 1,500 colonies on the plate and the chances are that a large proportion of them will appear as pin-point colonies. This is due to a restriction in their development by overcrowding.

It is a general custom in control work to plate 0.01 cc. of milk when examining pasteurized milk, and under normal conditions this is a satisfactory dilution. If, however, the count increases, this dilution will not be great enough and through overcrowding of the plates the colonies may

become pin point in size. It is assumed in this discussion that counts are made on standard extract agar with the proper reaction and incubated at 37° C. (98.6° F.) for 48 hours. If the reaction is too high or too low this in itself may cause colonies to become pin point in size. Incubation temperatures below 37° C. (98.6° F.) may prevent the appearance of pin-point colonies and slight variations in the composition of the medium may also exert an influence on their appearance. The appearance of pin-point colonies in plates, made with a standard medium and incubation, has been noted [178] from pasteurized milk, and it was found that the reaction of the medium had some relation to the appearance of such colonies.

The cause of pin-point colonies in general may be found in the composition of the medium, its reaction,[42] overcrowded plates, heat-resistant streptococci, or facultative or obligate thermophiles.[82] Temperatures of incubation [120] may play an important part in causing the appearance of pin-point colonies. There is no evidence that any of these organisms constitute a menace to health. It is of importance to know which is the cause and of particular importance to know whether the appearance of pin-point colonies is due to thermophilic or to heat-resistant streptococci or to other heat-resistant organisms which are not thermophilic, because the methods of eradicating the trouble will be different.

If due to a thermophile, the trouble is likely to be in the plant. If due to heat-resistant bacteria, present in large numbers in the raw milk before pasteurization, it is usually necessary to go back to the producing farm to locate the trouble. It can be quite readily determined whether thermophilic or non-thermophilic heat-resistant organisms cause the high counts and pin-point colonies. When high counts and pin-point colonies appear, a set of plates should be made and incubated at 50° C. (122° F.) for 24 hours. A count in this manner will at once detect thermophiles. Should no colonies or only a few appear at 50° C. (122° F.) it can be assumed that the high counts and pin-point colonies are due to non-thermophilic, heat-resistant bacteria. This can be verified by making standard counts at 37° C. (98.6° F.) of the raw milk and of the same milk pasteurized in the laboratory in sterile containers. The presence of a high proportion of a non-thermophilic heat-resistant type will be shown by a low percentage destruction of the bacteria by pasteurization.

Sterilization. Pasteurization must not be confused with sterilization. In the dairy industry the term sterilization is commonly used to signify a process which destroys vegetative cells but not spores. Thus, the sterilization of bottles and cans by treatment for a few minutes with steam not under pressure is not sterilization in the strict sense of the term. Non-spore-forming bacteria are destroyed at temperatures below 100° C. (212° F.) but spores require higher temperatures which may be as high as 121° C. (250° F.).

Sterilization of milk is usually employed in connection with evaporated milk rather than ordinary milk.

In the sterilization of evaporated milk too low a temperature or too short an exposure will permit the survival of the resistant spores of some

bacilli, and too high a temperature or too long an exposure will harm the quality of the product. A temperature of 116° to 118° C. (240° to 245° F.) for 15 minutes or longer is usually recommended[89, 108] but this temperature will vary with conditions. In the case of a "heat coagulable" milk, a "liver" or curd may form which can not be shaken out; and, if a low processing temperature is used, the product may be ruined by the action of surviving bacteria. Sometimes an organism with particularly resistant spores may get into a plant; and such high temperatures for sterilization may be required that it is impossible to shake out the resulting "liver." Too high temperatures may also harm the color and flavor of the product.

Some authors state that most of the cans of evaporated milk are sterile if they have been properly sealed and processed. Some workers contend,[186] however, that many cans of milk are not sterile, although comparatively few of them show growth under ordinary conditions.

Sterilization of milk in its original form is not practiced in this country although it has been done on a small experimental scale in an attempt to develop a market. In Europe, however, there has been further development along this line. The general principle employed is to heat the milk very rapidly to high temperatures sufficient to destroy spore-forming bacteria. It is no doubt possible to sterilize milk by this method but the greatest difficulty comes in placing it in containers so that it will remain sterile.

Influence of Pressure

Heat is the agent most generally used to kill bacteria in milk or inhibit their growth, but as even mild heating will cause changes in the milk constituents, other methods have been used and recommended for special purposes. When one considers the nature of the substances present in milk, their great chemical variety, and the delicacy of their colloidal relationships, it is obvious that any treatment which kills bacteria by a chemical effect, direct or indirect, on the cell protoplasm, must almost inevitably produce changes in the milk itself.

A treatment causing purely mechanical injury to the bacteria would perhaps be ideal, but thus far no practical method for this purpose has been devised for milk. Hite[81] has reported that milk subjected to a pressure of 90 tons per square inch kept without souring for two weeks. After prolonged storage the milk showed signs of deterioration as a result of the action of enzymes. Later Hite, Giddings and Weakley[82] found that non-spore-forming bacteria suspended in distilled water were killed by a pressure of 100,000 pounds per square inch without holding, or by a pressure of 35,000 pounds maintained for 150 minutes. Yeasts and some species of bacteria were killed by lower pressures.

Macheboeuf, Basset and Levy[103] have recently investigated the effect of pressure on enzymes. They found that enzymes are more resistant than vegetative bacterial cells, but less resistant than bacterial spores. Enzymes were inactivated at pressures of from 10,000 to 17,000 atmos-

pheres, depending upon the enzyme, its origin, the reaction of the medium, the chemical nature of the medium and the duration of exposure to the pressure.

In an atmosphere of carbon dioxide bacteria are much more readily killed by pressure than in the absence of carbon dioxide. Swearingen and Lewis [156] found that carbon dioxide under a pressure of 300 pounds per square inch killed most of the cells of a culture of *Escherichia coli.* Sudden release of the pressure did not increase the killing effect. Within certain limits change of the pH of the medium did not influence the effectiveness of gas. The controlling factor was the duration of the pressure. The killing was attributed to the precipitation of certain colloidal systems within the cell. This method of killing bacteria has never been applied to milk in large quantities.

Influence of Sound Waves

In recent years attention has been given to the destructive effect of high frequency sound waves on microorganisms. Wood and Loomis [177] found that paramecia, erythrocytes and cells of *Elodea* were ruptured when exposed to sonic waves with a frequency of 300,000 per second. Using a wave frequency of 406,000, Harvey and Loomis [78] obtained almost complete destruction of cultures of *Bacillus fisheri* suspended in sea water, but they concluded that the use of their method commercially for killing bacteria was not feasible because of the great expense.

Chambers and Gaines [85] have recently reported a method for killing from 80 to 100 per cent of the bacteria in raw milk, using a nickel tube vibrator with a frequency of 8,900. Their apparatus provided for continuous flow and could be expanded to handle large quantities of milk. There was no apparent reduction in the number of bacterial spores when the continuous flow was used, but when the outlet was connected by means of a rubber tube with the inlet and the apparatus used as a reflux, a large proportion of the spores of a culture of *Bacillus subtilis* was destroyed in fifteen minutes. These workers believe that with some improvements on their method it is not unreasonable to expect complete sterilization of milk without any change in the milk itself.

Influence of Electricity

Electrical treatment is theoretically more promising. Much of the early work was done on cultures of parasitic bacteria, by physicians interested in possible therapeutic applications. Zeit,[179] among others, brought out the fact that direct currents produced their effects partly by heat, but chiefly by the chemical action of the products of electrolysis. Alternating currents of low frequency rather favored bacterial multiplication, but currents of high potential and frequency definitely sterilized the bacterial suspensions he used, though with a strong odor of ozone. Other investigators have reported similar results. The subject is complicated by con-

siderations of the heat which is always produced when the milk is so treated.

Anderson and Finkelstein [2] investigated a process for milk purification using 2,300 volts, 14 amperes and a frequency of 25, which gave results comparable to those of pasteurization, without destroying the cream line. They concluded that the bacteria were really killed by heat, and that the apparatus furnished a method for producing a very sudden high temperature for a brief period, uniformly distributed since every particle of the milk must act as the conductor of the current. Chilson [86] evidently had a similar opinion about the electrical apparatus which he included in his comparative study of the efficiency of different types of pasteurizers.

Electrical milk purification has been thoroughly studied in England at Liverpool by Beattie and Lewis, who have published at intervals since 1913, and at Birmingham by Leith, with the collaboration of Sir Oliver Lodge. The Birmingham investigators inclined to the belief that the destruction of the bacteria was produced by heat rather than by the direct action of the current. Beattie and Lewis [24] have published the results of an investigation of this point under carefully controlled conditions. While the effect of heat could never be ruled out entirely, they satisfied themselves that the electrodes were not the source of the heat, but were warmed by the milk, and, as both *B. tuberculosis* and *B. coli* were killed in a few seconds at temperatures not over 64°, they concluded that, in the apparatus they used, the current itself, as well as the heat generated in the particles, acted as a germicidal agent.

Recent investigators have been more concerned with the efficacy of the method than with a possible electric effect distinct from the temperature rise. Devereux [54] reported 99 per cent efficiency in killing bacteria. Later, with Gelpi,[67] he showed that the electrical method at 71° killed 99.5 to 99.7 per cent of a suspension of anthrax spores, while the holding method (62.8° for 30 minutes) caused a reduction of only 0.3 to 2.7 per cent. Similar results were obtained with *B. subtilis, B. mycoides, B. mesentericus,* and *B. megatherium.* Subsequent experiments [68] indicated that the temperature attained within the spores was probably higher than that of the surrounding medium. They concluded that the lethal effect was due partly to the heat generated within the spores themselves.

Stabler's work [151] showed that the electrical process at 73° was as efficient in per cent and types destroyed as the holding method at 62°. She observed also that the rate of cream rise was somewhat reduced by this treatment, so that at the end of 24 hours, the cream layers were somewhat thicker than in bottles filled with the same milk, pasteurized by the holding method. Ultimately (144 hours) the cream layers were equal.

In several states the pasteurization laws have been modified or are now so interpreted that the electrical method is an accepted procedure.

Influence of Ultraviolet Light

The inhibitory action of sunlight on cultures of bacteria was observed by pioneer bacteriologists who soon recognized that the violet end of the spectrum was responsible. The invention of the quartz mercury vapor lamp has made it possible to study these effects quantitatively and also to use rays of wave lengths so short that they can not be received at the earth's surface from the sun, on account of atmospheric absorption. Coblentz and Fulton,[39] working with *Bacterium coli communis,* sprayed in a thin layer on agar plates, have demonstrated an abiotic range up to 3,650 Ångstrom units, with a lethal range below 2,800. The work of other investigators, with different organisms, is in good agreement with these figures. This destruction is not due to the formation of nitrous acid, ozone or hydrogen peroxide, but to a direct photodynamic effect upon the protoplasm. Bacteria so treated become granular and exhibit under the microscope evidences of incipient coagulation of the protoplasm or even bacteriolysis, if the exposure has been prolonged, according to Cernovodeanu and Henri.[34] Most proteins absorb portions of the ultraviolet end of the spectrum; Kober[96] working with peptones and amino acids has established definite bands in this region for tyrosine and phenylalanine, the others causing an indefinite general absorption. Bacteria and protozoa have been shielded from the destructive action of the rays by solutions of tyrosine and aminobenzoic acid in experiments carried out by Harris and Hoyt.[76, 77] The destructive action was confined to wave lengths from 2,480 to 2,710 Ångstrom units—the precise range absorbed by phenylalanine and tyrosine. They conclude that "the susceptibility of protoplasm to ultraviolet light is conditioned by the selective absorption of the toxic rays by the aromatic amino acid radicals of the proteins." These considerations explain the fact that ultraviolet light has been applied with far more success to the sterilization of water than to that of milk. Only clear water lends itself to this treatment, as any particles causing turbidity or opacity in a liquid are likely to be large enough to absorb rays of such short wave length. The effects of the dissolved and suspended materials in milk, and especially the specific action of the proteins present, in screening the bacteria from the rays of lethal wave length, necessitate exposure in very thin layers if any considerable reduction of numbers is to be accomplished. The work of Ayers and Johnson[7] showed that even with several minutes' treatment of very thin layers of milk, not all the bacteria were killed. A more serious objection was the very unpleasant taste imparted to the milk. This has been the experience of many workers. Römer and Sames[138] reported that the production of the undesirable flavor was accompanied by a lowering of the iodine number in cream and butter and destruction of the oxidase reaction in milk. Recent work on ultraviolet light treatment of milk has been done with a view to the increase of antirachitic substance present[152]—an effect which it is believed may be due to the action of the ozone formed.[160] This gain

in antirachitic vitamin is, however, accompanied by destruction of the fat-soluble vitamin A.[161, 181]

Influence of Osmotic Pressure

Bacteria must absorb their nourishment from solutions by osmosis. It is evident that any chemical or physical force which modifies the permeability of their membranes or the surface phenomena responsible for adsorption will exert a strong and possibly a determining influence upon their metabolism and multiplication. Such factors as osmotic pressure, salt effects, and surface tension are of great importance and are almost inextricably interrelated in their effects upon bacteria. When microorganisms are placed in solutions of high osmotic pressure, water is abstracted from them and the protoplasm appears shrunken and somewhat deformed. This "plasmolysis" does not necessarily kill the bacteria, but their multiplication may be completely inhibited. Molds and yeasts, however, are notably more resistant to such treatment. The preservative action of strong salt solutions is due partly to their high osmotic pressure, partly to the characteristic salt effect dependent upon the ions.

Sweetened condensed milk is not sterilized at any step in its manufacture. Its keeping quality depends on the exclusion of air, and the high osmotic pressure induced by condensation and addition of sucrose. The finished product contains usually about 45 per cent cane sugar, but the osmotic pressure will obviously be much greater than that of a 45 per cent aqueous solution. Mojonnier and Troy[108] give as a minimum standard 8 per cent fat, 20 per cent milk solids-not-fat, 44.5 per cent sucrose, 27.5 per cent water. Of the 20 parts milk solids-not-fat, 10.9 are lactose and 2 are salts. It is obvious that even if all the water were available for the sucrose, this would be about a 62 per cent solution. As a matter of fact there are present considerable amounts of hydrophilic colloids to compete with the sugar for water. To this osmotic pressure must be added that of the lactose present, which exerts a pressure equal to that of an equal amount of sucrose, and the salts which on the basis of their molecular weights can be shown to exert a pressure equivalent to that of 4.35 times their weight of sugar, and more if ionized. The resultant osmotic pressure would be greater than that of a 75 per cent solution of cane sugar, a decidedly unfavorable environment for bacteria. Molds and yeasts are able to grow on this medium and are a fairly frequent cause of spoilage, particularly if air has not been completely excluded. It is a matter of common observation that condensed milk left in contact with the air will support the growth of a dense felt of mold on its surface. The "buttons" investigated by Rogers, Dahlberg and Evans[181] are due to the growth of mold which ceases as soon as the small amount of oxygen present is exhausted. Yeasts and yeast-like organisms are not so readily inhibited by exclusion of air, and even those that require oxygen for multiplication are able to cause serious spoilage in its absence, if present in large numbers, for fermentation is favored by the absence

of air. Hammer[78] reported an outbreak of fermentation in a condensed-milk factory due to a torula. Savage and Hunwicke[136] isolated yeasts of various kinds from the majority of the blown tins which they studied. The bacteria which survive the process of manufacture and the sugar treatment are not those characteristic of raw milk. Members of the colon-aerogenes group have been reported, but never in large numbers or as a cause of serious spoilage. Anaerobic rods are evidently controlled by the sugar. Spore-bearing aerobes can usually be cultivated by inoculating suitable media with condensed milk, but they do not multiply in the canned product. Many of them are obligate aerobes; moreover, they are probably present as spores which have survived pasteurization or have fallen in from the factory dust. There is good evidence that solutions of high osmotic pressure prevent the germination of spores. Both Eijkman[59] and Curran[46] demonstrated a rather definite limiting osmotic pressure for germination. In the absence of other limiting factors germination of spores is inhibited within a range corresponding roughly to 36 to 46 atmospheres. Micrococci are apparently always present in sweetened condensed milk; Savage and Hunwicke stated that they had never found a sample of sweetened condensed milk which did not contain them. Evidently they multiply for a short time after the cans are sealed, and then die out. Downs[58] has isolated two strains capable of producing thickening but as a rule the micrococci produce no deleterious effects.

Salt Effects

Physiological processes in general are profoundly influenced by the presence of salts which determine the stability of colloidal dispersions. The ions into which the salts dissociate may affect the equilibrium of a system in a variety of ways. One group of phenomena depends upon the chemical peculiarities of the ions—such as the precipitating action of salts of heavy metals upon proteins, in which the anion is relatively unimportant. Adsorption, surface action and diffusion through membranes, so essential to cell nutrition, are determined by the electrical charges carried by the ions. This is noticeable in dilute solutions, and is naturally most marked with multivalent ions. The peptizing action of certain salts upon suspensions, and their influence upon imbibition and hydration of proteins have made it possible to arrange certain physiologically important anions and cations in the so-called lyotropic series. No one knows the cause of this orderly gradation of potency, manifest upon a variety of phenomena apparently unrelated one to another, but it has been best explained as due to a change which the ions produce in the state of the solvent itself. These summarizing statements indicate the complexity of the processes which must be induced or modified by the dissociation of the electrolytes present in a culture. For a full and authoritative discussion of the subject the reader may well consult Freundlich[64] or Bayliss.[23] The salts present in the medium surrounding the organism may affect the food materials, rendering them more or less susceptible to attack by enzymes; they may

affect the permeability of the ectoplasm to nourishment from without or end-products from within; finally they may profoundly alter the life processes which go on in the protoplasm itself. This may result in a change in the rate of multiplication or in a modification of metabolic activities such as fermentation.

Holm and Sherman [88] have shown that multiplication of *Bact. coli* is stimulated or retarded by anions and cations in an order resembling that of the lyotropic series, and that those salts which accelerate growth widen the favorable range of pH. As early as 1892, Richet [126] had reported that, even with toxic metal salts, doses too minute to curb growth stimulated lactic fermentation.

Fabian and Winslow,[61] in reviewing previous work at Yale and elsewhere, stated that all the cations seem to stimulate growth and increase cell-permeability in low concentrations, and to have an opposite effect in higher concentrations. "Supposed specific effects are due merely to the fact that different cations have different quantitative potencies; and antagonistic or additive effects will appear according to the quantitative relationships involved." Their observations of anion effects on *E. coli,* with $NaOH$, $NaCl$, $NaHCO_3$, Na_2CO_3, Na_2SO_4, Na_2HPO_4, NaH_2PO_4, Na_3PO_4, and mixtures of $NaOH$ and the other salts mentioned, indicated that they all had the same general quantitative effects. Stimulating actions were a function of the Na content, 0.001 to 0.020 molal being the favorable range. The inhibitory effect of higher concentrations resolved itself into two factors, the Na content and the pH. Maximum stimulation occurred at 0.10 molal Na and a pH of 7.5, while Na concentrations above 0.6 molal and any pH above 8.3 were distinctly inhibitory. According to these workers, "salt effects" are due to three factors:—cation concentrations, all cations being quantitatively alike and differing only in degree of potency; pH, which, with a given cation, is determined by the nature of the anion; and an apparently specific stimulating effect of the phosphate ion, provided the other two factors are favorably adjusted. A study of the quantitative differences or "specific potencies" of cations [174] showed that in both the stimulating and the inhibitory zones roughly constant relationships were maintained and that, if the potency of Na be taken as 1, the series studied arranged itself in the order—K, 1; Li, 3; Ba, 5; Mg, 9; Ca, 12; Mn, 400; Zn, 700; Cd, 3000. It was concluded that some, but not all, of the phenomena of so-called salt-antagonism can be explained as simple additive effects of the specific potencies concerned.

Borman's investigation [81] of natural and acquired resistance to cations in certain strains of *E. coli,* yielded a slightly different sequence of ascending order of bacteriostatic and bactericidal potencies,—K and Na, Ca, Mg and Ba, Cu, Fe and Pb, Hg. This order was the same for all strains used, and when a particular strain acquired an increasing resistance to one cation, it became more resistant to all the other cations, thus confirming the idea of the qualitative similarity of cation effects.

That the salts present in liquid milk produce a resultant effect favorable to bacterial growth is obvious from the fact that it is one of the most

generally favorable media. In butter and cheese, NaCl is used to inhibit multiplication as well as for flavor. The course of Cheddar-cheese ripening will not be normal in the absence of salt; on the other hand, the eye culture which produces the characteristic flavor of Swiss cheese is completely inhibited by salt, which must consequently be applied only at the surface.

Influence of Surface Tension

Many surface tension depressants are so toxic to protoplasm that their effects on surface action are masked by their purely chemical reactions. Other substances formerly thought to have little or no chemical action upon bacteria are now known to be very toxic for many species. Sodium ricinoleate used by Larson and his coworkers [99, 100] belongs to the latter group.

Bacteria may respond to changes in surface tension in two ways: by ceasing to grow at all, if it is too unfavorable; or, by growing only in those portions of the culture where a favorable state of affairs is found. Surface tension depressants tend to accumulate at the surface of a liquid, consequently an organism in a medium with a tension somewhat too high for its most rapid growth will multiply chiefly or only near the surface. This has its bearing on pellicle formation, which can sometimes be prevented by addition of soap. Organisms of the colon-typhoid group which grow diffusely in broth (56 dynes) tend to accumulate at the surface of a synthetic medium with a surface tension approaching that of water (73 dynes). As pointed out by Day and Gibbs [50] surface tension variation without radical chemical change presents some difficulty. In a great many of the reported studies upon the influence of surface tension on bacterial growth the chemical nature of the depressant was the effective factor. Undoubtedly the best means of determining the effect of surface tension as the variant is to employ a sufficient number of well selected depressants so that the effect of each may be recognized. Sodium oleate seems to be one of the most satisfactory surface tension depressants though its value may be limited in the presence of acid. Ayers, Rupp and Johnson [19] in an attempt to differentiate streptococci by this method, observed that *S. pyogenes* was the most susceptible to lowered surface tension and *S. lactis* the least. Frobisher,[65] working with a number of pathogens, has observed characteristic differences in resistance to the action of sodium oleate.

Contrary to a formerly held belief it is now established [48, 50] that *Lactobacillus acidophilus* cannot be differentiated from *L. bulgaricus* on the basis of growth at different surface tension. Surface tension seems to have little influence upon the germination of bacterial spores. Curran [47] found that reduction in the surface tension of the medium representing 15 dynes produced slight effect upon the germination of the spores of *B. mycoides.*

Influence of Desiccation

Milk powder is never sterile yet its quality is rarely, if ever, impaired by bacterial action. The principal causes of spoilage are oxidative changes

in the fat, in which microorganisms play no part. On the other hand it is possible, and even commercially profitable, to prepare dried cultures of bacteria, particularly lactic acid starters, which remain alive for months. These apparently contradictory phenomena arise from the fact that in conditions which favor rapid growth, cultures soon die out; while circumstances which suppress active vegetative metabolism cause a state of suspended but prolonged animation. This accounts for the exasperating ability of undesirable organisms to remain alive in thin films of organic material left to dry in the air and in the dust of barns and factories. During the process of desiccation the concentration of the substances present is progressively increased and the bacteria may be subject to harmful amounts of acid or salts. The presence of proteins, starch or lactose is a protection against these agencies which at most are inhibitory rather than lethal. Rogers [130] has shown that in drying milk cultures, the stage when there is 5 to 10 per cent water present, is the most fatal to bacteria. Rapidity of drying shortens this period of danger, and hastens the state when lack of free water prevents injurious reactions. A low temperature during the drying and subsequent storage minimizes biochemical activity and favors survival. The Bureau of Dairy Industry prepares, for distribution in the field, dried cultures of *Propionicibacterium shermanii,* the organism largely responsible for the characteristic flavor and eye formation in Swiss cheese. Sterile skim milk inoculated with broth cultures of the organisms are dried by the spray process. The age of the culture, preliminary temperature of incubation of the culture, and the temperature at which powdering occurs, are carefully controlled. The final product may contain as many as 700,000,000 viable bacteria per gram. These dried cultures have largely displaced the liquid culture formerly used as they are much more easily handled and the organisms contained therein retain their viability for a much longer time. Similar drying processes have been applied to certain strains of *Streptococcus thermophilus, S. lactis,* and *Lactobacillus bulgaricus.* Fair results were obtained with the first two of these organisms.

Recent years have seen the development of a large number and variety of powdered infant foods. Hucker and Hucker,[88] Mattoon [106] have studied the bacteriology of these products. The former investigators found no appreciable numbers of hemolytic or viridans streptococci in an examination of 200 samples of prepared infant food. Mattoon [106] found that the bacteriological counts obtained from 20 kinds of powdered infant foods ranged from 36 to 98,000 per gram. The majority found were spore formers. Viridans and *beta* hemolytic streptococci were found in five different products. Dick and Dick [55, 56] conducted an investigation of powdered protein milk in connection with a study of an epidemic enteritis, surviving *beta* hemolytic and viridans streptococci were regarded with suspicion as the possible causative factor.

Delepine,[52] studying milk powder, found that most of the non-spore-bearing bacteria in milk were killed during the drying process. This was largely due to the heat employed but not even the drum method which

prolonged the heating would kill them all. Factory contamination during pulverization and packing was responsible for a large proportion of the bacteria present in the finished product. In addition to spore-bearers of the mesentericus type he found cocci of various kinds, a few small bacilli and several varieties of streptothrix. During storage the bacteria decreased somewhat in number. Coutts [43] confirmed and extended these observations. He stated that the colon type were usually absent from the powders he studied, but that streptococci were fairly frequent, as was also *B. enteriditis sporogenes*. Still the milk powders were in good condition, and the bacterial counts were low compared to those of fresh raw milk. Supplee and Ashbaugh [154] studied a series of milk powders made by the drum process with moisture contents from 1.74 to 9.34 per cent and with initial counts of from 750 to 100,000 per gram. Counts taken at intervals of two months showed that, at the end of the year, all the samples contained about the same number of bacteria—a few hundred per gram—regardless of the initial count or per cent of moisture, but the powders with low counts when fresh reached the minimum sooner. None of the samples showed any deterioration caused by bacteria. Later Supplee and Bixby [155] reported that the Just roller (drum method) destroyed all hemolyzing streptococci including *S. epidemicus*, enterococcus, a green producing streptococcus, and the Morgan dysentery bacillus. The total bacterial count and the total count of hemolyzing colonies were found to be much higher in the milk powder prepared by the spray process than in that made by the drum method.

Hunwicke and Jephcott [90] report that *B. tuberculosis* was either destroyed or rendered avirulent to guinea-pigs in their experiments with the roller process and that this method is capable of destroying all non-sporing bacteria.

Influence of Carbon Dioxide

The use of carbon dioxide has been advocated from time to time as a method of improving or preserving the quality of milk or milk products. Procedures in which butter is churned or ice cream frozen in carbon dioxide at atmospheric pressure can obviously have no great effect on the bacterial content, except by the mere exclusion of air during that stage of manufacture. Such air-borne (or rather dust-borne) contamination is negligible compared to what the cream undergoes before it is put into the machine. Carbonation under pressure has been widely used in preparing beverages. Koser and Skinner [98] have shown that the acidity thus produced is chiefly responsible for the disappearance of bacteria since pH values as low as 4.0 are sometimes attained. If well buffered liquids were used the bacteria died out more slowly than in plain water. The abundance of buffers in milk make it unsuitable for successful carbon dioxide treatment, on theoretical grounds. Prucha, Brannon and Ambrose,[121] like other investigators, found that while the rate of multiplication is retarded the numbers of bacteria present steadily increased. Even at 60 pounds pressure the count in one sample rose from 35,000 to 359,000,000 in four

days. The present status of the carbonation of dairy products is given by Prucha, Brannon, and Ruehe.[122]

Influence of Preservatives

The use of chemicals to preserve milk intended for human consumption is prohibited by law for obvious reasons. However, samples for analysis may have to be kept for some time and a brief consideration of chemical disinfection and the suitability of the compounds usually employed will not be out of place.

It has been pointed out that any substance toxic to bacteria by virtue of a chemical effect on protoplasm will in all probability react with some of the ingredients of milk to which it is added. This may impair its efficiency against the organisms present or perhaps may interfere with the accuracy of subsequent analysis. The oxidizing action of hydrogen peroxide or sodium hypochlorite is never as effective in milk as in water. They have both been advocated as emergency preservatives for milk intended for food, since small quantities would not be injurious to the consumer, but they are not adequate to keep it for any length of time unless used in amounts which cause a bad taste. Hypochlorite solutions are widely used in cleaning milk plant equipment which can not be sterilized by heat. The efficiency is proportional to the available chlorine, and, as it is reduced by the presence of milk or slime, thorough rinsing before treatment is necessary to get the best results.

Toluene and chloroform have both been used with some success to retard bacterial multiplication. However, they are not equally effective against all organisms; they do not greatly interfere with enzyme action, a circumstance which renders them indifferent preservatives; and they soon evaporate.

The Methods of Analysis of the Association of Official Agricultural Chemists (1925) recommends the use of potassium bichromate, bichloride of mercury or formaldehyde. Potassium bichromate treatment always causes an error in the total solids determination. Magnier de la Source[105] stresses the slowness with which this substance diffuses in milk. Hinard[80] found that in his experience 0.1 per cent merely retards bacterial growth. He states that the error in total solids is due to such variable reactions that he considers it impossible to correct for them mathematically.

Mercuric chloride is the most toxic of the salts of heavy metals. It is particularly useful in preserving composite samples for Babcock testing, as Jackson[91] pointed out, for it prevents curdling and does not alter the fat. The fact that it acts chiefly by precipitating albumin makes it obviously unsuitable when careful protein studies are to be made. In such a case the casein nitrogen figures will be too high, and the albumin nitrogen too low.

Formaldehyde also combines with proteins though its poisonous effect upon bacteria is probably not limited to this ability alone. Porcher[119] found that one drop of a 40 per cent solution in 50 cc. of milk does not

affect the reductase test, but that any larger quantity would interfere. The hardening of protein by formaldehyde is of great usefulness in preserving anatomical and botanical specimens, and in the manufacture of casein plastics, but naturally causes some error in analyses of milk to which it has been added. After a careful investigation, Palmer and Eckles [118] recommended it in 1/1700 to 1/1250 dilutions with the warning that heat coagulation can not be used on such a sample to determine albumin. They call attention to the fact that none of these preservatives can give entirely satisfactory results unless it is added while the milk is fresh,—that is, not more than 24 hours old at the most, particularly in warm weather. Otherwise the bacteria will have grown sufficiently to liberate proteoclastic enzymes. These may continue to cause protein degradation although the bacteria themselves have been destroyed.

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Chapter XIII

Yeasts and Molds of Milk and Milk Products *

Introductory. Molds and yeasts, in addition to bacteria, are constantly present in dairy products and are frequently active agents in the changes encountered. All of these groups are technically plants—fungi, in the largest sense of the term. They have many characteristics in common; they are devoid of the coloring matter of the green plants (chlorophyll), hence are dependent for their energy for growth upon the disintegration of complex compounds mainly of organic origin; they differ from the green plants also in the nature of their cell-walls. Direct rays of the sun are inimical to most of them instead of being essential. The green plants are found in the open, where their surfaces may be reached by fresh air and sunshine; the fungi in their actively growing phase may be completely submerged in the substratum. They frequent the murky or even dark corners and some of them obtain their needed oxygen either from concentrations of free oxygen entirely too low to support green plants, or from the actual disintegration of complex compounds.

Yeasts. The yeasts belong to a group of fungi with comparatively simple vegetative structures as seen under the microscope. They differ from the bacteria (see preceding chapters) in presenting plant cells in which cell-wall and protoplasmic mass are definitely distinguishable. In preparations of living yeasts, cytoplasm, walls, vacuoles, oil globules and various types of granules are visible. By special staining methods nuclei are demonstrable. The yeasts multiply by budding rather than by fission, hence several cells in various stages of development commonly remain connected when growing cultures are studied. Typical yeasts differ from molds in failing to develop complex filamentous networks or mycelia, consisting of branching vegetative hyphae. Border line forms occur, however, which when grown under one set of conditions appear as yeasts, and under another set of conditions assume the mycelial form. Spores when produced are developed within old yeast cells and are characteristic in number and form in the various groups.

Many genera and species of *Saccharomyces,* as the group is called, are found on normal and fermenting cattle feed. Ensilage contains large numbers to the gram in normal condition; the numbers become enormous when ensilage is exposed to the air even for periods of a very few days. The air of the stable, feed troughs, gutters and manure piles are well seeded with them, hence yeasts are found well distributed in dairy products. Hastings [12] has shown them to be present in certain types of Swiss

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cheese with off-flavors. They are abundant in the surface slimes on Camembert, Brie, d'Isigny, Limburger and related cheeses. Yeasts have been found as factors in the production of fermented milk products under such names as Kefir and Kumys. Hammer [10, 11] described one of them from sweetened condensed milk as *Torula lactis-condensi* and in another paper described *Torula cremoris* and *T. sphaerica* as factors in the "yeasty" spoilage of centralizer cream. (See also Hunter, 1918.[14])

Aside from large groups classified by the production or failure to produce spores under specified conditions and by certain outstanding differences in size and shape, the identification of yeasts rests principally upon fermentation tests. The ability of yeasts through their enzyme, zymase, to produce alcohol from various sugars has so far overshadowed their other activities industrially that their constant presence and participation in other fermentations and decompositions is commonly overlooked. Their invariable presence in dairy products,[22] however, has directed investigations into their presence and significance which will eventually add much to our general knowledge of this group of microorganisms. For specific information such standard references as Jorgensen's "Micro-organisms and Fermentation," or Guilliermond-Tanner's "The Yeasts," are available.

Actinomycetes. Colonies of the *Actinomyces* group grow slowly in the usual culture media used in dairy work. Most of them are counted as bacteria in plates incubated only 24 to 48 hours. After about a week these colonies commonly appear as hard fibrous masses rather deeply set in the substratum with only the centers rising just above the surface as white or bright-colored "cushions." Viewed with the compound microscope as they stand in the medium, these aerial growths consist of fine tangled felts of filaments, or hyphae, mostly 1μ or less in diameter with more or less specialized spores in a few species, often showing very beautiful spiral forms. When mounted for study under higher magnifications, these aerial hyphae commonly break up into separate cells which resemble bacteria very closely. The submerged portion of the colony, however, consists of a complex network of branching vegetative hyphae, thus allying the group closely with molds, although they have been most commonly studied by bacteriological methods and described from their reactions instead of upon the morphological data used in describing fungi.

Members of the *Actinomyces* group are present in all cultures from raw dairy products. Fellers [7] in New Jersey found them as a cause of bad flavors in milk. Others have shown them to be present in butter. They are regularly in cultures from cheeses of different varieties. The possibilities of the group as a cause of deterioration in dairy products are only beginning to be understood as the studies of Waksman,[34] Krainsky,[15] Conn,[4] Drechsler [6] and others have made methods of isolation and identification better known. For the description of species the special literature of this group must be consulted.

Molds. The term mold is applied loosely by common usage to an indefinite aggregation of species representing widely separate groups of

fungi. The common factor in this aggregation of forms is the development of more or less conspicuous fibrous, or felted growth, the *mycelium* (plural *mycelia*) upon the surface of substrata or, if submerged in substrata, still presenting the evidence of masses of fibers or filaments (each filament is called a *hypha*) with or without conspicuous fruiting organs. This description is loose enough to include all of the several hundreds of hyphal fungi reported by various workers as found in the dairy.

Any species of mold growing in pure culture produces a characteristic colony which is usually composed of a branching felt of vegetative filaments from which some sort of fruiting organs develop in the later stages of growth. Upon natural substrata many species are usually so mixed that identification of the particular species present is usually impossible until each form has been isolated and pure cultures of it have been grown.

The vegetative mycelium may consist of a complex system of branching but unseptate tubes as in the *Mucors* within whose growing hyphae rapid circulation of the protoplasmic mass may be readily seen with the microscope, or of a similar system of filaments consisting of cells placed end to end and remaining thus permanently connected. The vegetative hyphae are, for the most part, submerged in the food substance or closely appressed to its surface. In some species, however, part of the hyphal network rises above the surface to produce a vegetative aerial mycelium giving a felted, cottony or *floccose* appearance to the colony with spore-bearing branches borne upon the aerial hyphae. In other cases only the spore-bearing hyphae rise above the surface producing the appearance of velvet (velvety) or of a miniature field of grain, or giving a powdery appearance from an abundance of spores produced and commonly so loosely attached to stalks as to become easily detached.

Any vegetative cell from a mold colony, if removed and planted in a favorable medium,[85] will commonly reproduce the characteristic appearance of the species. The mycelium growing in or upon the surface of the substratum commonly spreads very rapidly, thus multiplying greatly its capacity for decomposition and fermentation. In the more common molds of the dairy the usual method of propagation, however, is by asexual spores, which are produced rapidly and in enormous numbers. These asexually produced spores commonly called *conidia,* are more or less specialized reproductive cells produced upon more or less differentiated branches, the conidiophores,[8] or in spore-producing sacs called *sporangia* borne upon specialized stalks or sporangiophores; or within special complex bodies known as *pycnidia.*[4]

Sexual or so-called "perfect" fruit appears in various forms varying from simple fusions of two conjugating cells without accessory structures to very complicated fruit bodies. Elaborate systems of classification of the fungi are based upon the mode of development and structure of these various spore-producing organs and of the spores themselves, thus making possible the close and accurate descriptions necessary to separate and identify many thousands of species. For such taxonomic systems, reference to works upon fungi is necessary since the systems are too elaborate

to be discussed here. These "perfect" forms are only occasionally encountered in cultures as made in the dairy laboratory.

Many investigators have collected and described the various species of molds which could be isolated from the dairy and its products. Recently Bisby,[1] Jamieson and Timonin have tabulated the occurrence of 104 species as reported in their own paper, compared with the reports of Macy[18, 19, 20] in Minnesota and of Grimes[5, 8, 9] and his coworkers in Ireland. Only three of these species were found in all three groups of studies, namely, *Alternaria sp.*, *Cladosporium sp.*, and *Oospora lactis.* Agreement in identification and the use of names in these lists was secured since the three collections passed independently through the hands of Thom. Aside from recent literature several hundreds of species names have been listed in the literature of the past half century. Very few of those forms have been reported frequently enough or sufficiently intensively studied to establish even presumptive significance as organisms of the dairy.

Significant species. Scrutiny of lists of this kind thus leads clearly to the recognition of species significant to a process or a product as those forms which by their activity become responsible for measurable or definable biological changes. A consideration of the sources of all these organisms is, therefore, necessary. In presenting a brief consideration of these molds only those forms which bear some relation to dairy practices or which occur very frequently are discussed here. For the purpose of aiding in the identification of these fungi a dichotomous key has been prepared, in which lines of separation have been drawn among the species deemed significant and certain other organisms which occur frequently. Necessarily many species will be occasionally found for which no clues to identification can be included.

Sources of molds and yeasts. Mold spores and yeasts are exceedingly small, rarely more than a few thousandths of a millimeter in diameter. These spores and yeasts are found in and on feeds, manure, soil, decaying vegetable and animal matter of every description. They are very light, hence they float readily in the air and form a portion of the fine dust which is always present in the atmosphere and which is increased greatly by air currents, by the movements of stock, by the handling of feed, by the cleaning of the stables and of the cattle themselves.

Milk thus becomes contaminated with yeast cells or mold spores of any or all the species growing in the soil, upon the forage or feed used, upon or in the manure or other waste products always more or less present, or carried by man or any of the domestic animals into the milk-producing establishment. Many of these species are entirely incapable of growing normally and reproducing in milk. If such species germinate at all, they usually die quickly without affecting the condition of soundness or food value of the milk. Some forms grow feebly in milk, but sufficiently to produce stale odors and abnormal taste, if present in large numbers and permitted to grow for fairly long periods. Some species of molds are capable of growing freely and richly in milk with consequent

rapid spoilage or fermentation of the product. Only a very few of this total number of forms are so well adapted to growth in milk and its products that they colonize in and about dairy establishments and are constant factors in the fermentations encountered, thus furnishing the species significant in the dairy and worthy of discussion within our limitation.

In all of this consideration it must be remembered that molds and yeasts like the bacteria present are not a part of milk, even though they are always present to some degree, because it is practically impossible to produce milk without some exposure to the air, to the hands of the milker, to the skin of the cow, or to contaminated surfaces in the utensils used. Unlike bacteria, molds and yeasts are not commonly found in the udder, hence aside from the special case of the udder infected with a mycotic disease, molds and yeasts are contaminations which enter only after milk has been drawn.

Factors affecting activities of yeasts and molds. The abundance and importance of these organisms calls for brief consideration of their physiological activities as a basis for their prevention or utilization under dairy conditions. Nutrient values have already been discussed. Among the major factors favoring or limiting the activities of yeasts and molds, oxygen, temperature and moisture are probably the most important.

Oxygen relations. Molds are mostly aerobic, although a few species are capable of growing where the concentration of oxygen is materially less than that of the atmosphere. As encountered in dairy products, molds grow readily in the surface layers of such liquid products as milk and cream, while but few and scattered hyphae are found deep in the liquid mass. A few forms such as *Oidium* will penetrate rather deeply into semi-solid masses and a few other forms such as the Roquefort mold (*Penicillium roqueforti*), follow cracks and open spaces through whole cheeses although they actually penetrate only slightly into the solid cheese itself. Oxygen in such cases seems to be the limiting factor. Most of the species studied follow cracks or holes to very short distances into such masses as cheese or butter, although they may form heavy felts of mycelium upon the surface.

Moisture. Molds and yeasts are very dependent upon available moisture for their growth and multiplication. Yeasts have adapted themselves to growth in liquids, hence commonly thrive in fluid substrata. Molds while growing readily on the surface of liquids of favorable composition, commonly find their optimum development in solid or semi-solid substrata. Very few molds are able to compete successfully with yeasts and bacteria in liquid cultures and many of them are overgrown and suppressed in such situations. Among the molds themselves there are great differences in the amount of water necessary for optimum or most rapid and characteristic growth. As a result, series of species are encountered in the study of dairy problems as more or less closely associated with particular products either as causes of deterioration or loss, or as agents in ripening changes desired. Many molds will grow vigorously in pure culture upon

milk but comparatively few of them can develop upon milk in characteristic form unless the competition of bacteria and yeasts is reduced or eliminated by sterilization or by acidification before inoculation. Similarly upon such moist cheeses as cottage (about 75 per cent moisture) or whole milk Neufchatel (about 50 to 60 per cent moisture) bacterial and yeast activity often interfere with mold growth except in the dominant species *Oidium lactis,* which is regularly found in close association with bacteria. Molds tend to grow richly upon cheeses carrying from 35 per cent to 50 per cent of moisture. Below 35 per cent of moisture the tendency to develop mold diminishes rapidly until at 30 per cent very little mold develops. The restricting factor in moisture content is not the absolute percentage of moisture present but the water available to the mold. In cheese, few molds are able to extract water for growth when the total amount falls as low as 30 per cent. Molding quickly begins if the percentage exceeds 30 per cent. In butter, the fat is inert, hence the limiting factor is the concentration of solutes in the water present. These examples fairly illustrate the cause of the absence or development of mold in many situations in the dairy and upon its manufactured products.

Temperature. Temperature is a major factor in the rate of mold development. Molds for the most part, reach their optimum rate of growth somewhat below blood heat or 37° C. (98° F.). Temperatures of 30° to 33° C. (85° to 90° F.) are favorable to moldiness. From such a point of most rapid molding there is a progressive reduction of mold growth with its deteriorative effects as the temperature falls toward the freezing point. This rate of slowing up varies, however, with the species of mold so that some species, especially certain green molds of the dairy, are found to continue their activities to much lower temperatures than other food-contaminating forms. Total inhibition of such activity seems to be reached only when the moisture present in the product is actually turned into ice crystals. In some products, therefore, definite development of molds has been shown to occur at several degrees below the freezing point.

In addition to these determining factors, the salts, sugars, and proteins present in particular substrata favor some organisms and restrict others; for example, molds and yeasts are commonly very dependent upon assimilable sugar but many of them find milk sugar unavailable for nutrition. Such species tend to grow poorly upon milk products.

Mold prevention. Taking advantage of the physiological characteristics already discussed, mold injury to dairy products may be controlled by establishing such temperatures as will prevent or minimize their activity during the time necessary for marketing the several products. Choice between the ice refrigerator and the cold storage will be influenced by the product to be preserved and by the period of storage necessary.

The same end is accomplished by desiccation in products such as milk powder, in which the available moisture is reduced below the percentage necessary for mold growth.

Exclusion of oxygen as a factor in preservation presents many diffi-

culties in obtaining practical results. The amount of oxygen necessary to permit certain organisms to grow is very small and the diffusibility of objectionable by-products of growth is very great. Nevertheless, closed containers, close-fitting wrappers, paraffin coverings and many similar means have been more or less successfully utilized to aid in lengthening the commercial life of dairy products.

The specific relations of certain molds to the dairy may best be considered product by product.

Milk and cream. Yeasts vary greatly in their ability to grow in milk and its products, but a group of lactose-fermenting yeasts has been described by various investigators. Yeasty cream [14, 17, 21] is not uncommon where cream for churning purposes is neglected, allowed to become stale or held for a considerable time at high temperatures. Yeasts also have been found in off-flavored cheese and consistently found objectionable. Fellers [7] in New Jersey traced an outbreak of taint in milk to a species of *Actinomyces;* others have repeatedly found these organisms. Market milk, unless old and very stale, rarely shows mold colonies or masses of mycelium. *Oidium lactis* is a common and offensive contaminant which develops mostly in the cream layer of old milk. It becomes a serious cause of deterioration when cream is brought together in lots for long-distance shipment. Accompanying *Oidium, Cladosporium* and the racemose *Mucors* are exceedingly abundant in many samples of cream. Many other species can be found occasionally by extensive plating from these products.

Butter. Mold as a factor in losses of butter takes two forms, a discoloration which produces a defect in appearance [2] but may or may not affect the flavor seriously, and a growth within the mass which is usually very destructive to flavor [23] without always affecting the appearance. Thus to stop all growth in butter with about 15 per cent moisture and about 3 per cent of salt, the product must be chilled to a temperature which will freeze brine containing 15 to 16 per cent of salt. On the other hand, a product like cheese, which can not be frozen without damage, may continue to suffer from some mold activity throughout the storage period. The surface injuries are mainly due to green molds of the genus *Penicillium,* some of which are sufficiently tolerant of fairly high salt concentrations to grow and fruit freely in the mixture of milk serum, water and salt on the surface of the butter, and in the wrapper and the wet inner surface of the container.[24] Other salt-tolerant forms are found among the species of *Cladosporium* and *Alternaria.* Some of these molds are also able to break down butterfat and casein with the production of highly flavored end products which diffuse rapidly throughout the package.

In contrast to these surface molds whose presence is visible, unsalted or mildly salted butter is often injured by the rapid growth of *Oidium lactis* throughout the mass. This mold is practically universally associated with the handling of milk and its products so that its presence in any raw material must be assumed,[21, 30] although it may be greatly reduced. It is, however, readily killed by pasteurization,[31] hence is susceptible of control by proper handling. The hyphae and spores when abundant in the cream

are still readily demonstrated by microscopical methods while damage due to the enzymes present is not excluded by ordinary pasteurization temperatures. It is also prevented from growing by brines of fairly high concentration, thus its activity becomes negligible in highly salted or in moderately salted butter.[13]

The surface molds are less readily controlled in the finished product, since the entire exclusion of infection from the air, the tools, and operations necessary is scarcely possible. The trouble may be minimized by proper selection and treatment of wrappers and containers, and by the exclusion of air.

Cheese. Milk at about 87 per cent moisture decomposes mostly as a result of bacterial activity with yeasts as an occasional and secondary factor. Cottage cheese at, perhaps, 70 per cent moisture becomes overrun with *Oidium lactis* as a dominant agent of fermentation with abundant bacteria, yeasts, and occasional *Mucors*. A whole series of skim-milk cheeses ripened to varying degrees shows a soft, wrinkled surface layer predominantly *Oidium* covering a glairy semi-liquid layer of partially digested casein. As the water content in these forms falls toward 50 per cent, bacteria are somewhat restricted and other molds become abundant. At water content between 50 and 60 per cent, under proper handling, the Camembert mold (*P. camemberti*) and its white variety (*P. caseicolum* Bainier) [33] become the dominant agents of ripening,[27] as on ripened Neufchatel, Camembert and Brie. When these cheeses are carefully handled the proper molds will suppress all other species. Contaminating species creep in when the cheeses are made with too low water percentage. Upon the surface of overripe or badly handled cheeses of the Camembert-Brie group, areas of yellowish brown or yellowish cream color mark the development of the *Penicillium brevicaule* [28] group of species or varieties whose growth is always accompanied by strongly ammoniacal odors. Green or blue-green areas indicate the invasion of other molds which frequently impart bitter flavors to the cheese. Laxa [16] described Ellischauer as a cheese related to Camembert made in south Bohemia and showing two molds as agents of ripening, *Penicillium album* (apparently *P. caseicolum* Bainier [33]) and a green species described as *P. nalgiovense* Laxa.

In the Roquefort-Gorgonzola-Stilton group of cheeses the open spaces in the interior of the mass are commonly lined with green mold belonging to a series of strains or varieties described by Thom as *Penicillium roqueforti*.[26, 28] It is also variously listed in the literature as *P. glaucum, P. aromaticum* of Sopp. This species has been shown to be the agent responsible for the characteristic flavor of a whole group of cheeses. *Penicillium roqueforti* is also the usual mold in deep narrow cracks in other cheeses, but other green molds are frequently found to follow such cracks into the mass far enough to injure the character of the product without giving any suggestion of the flavors associated with Roquefort, which has been shown by Currie to be due to the effect of *P. roqueforti* upon the fat itself. Varieties of this species are very abundant in soil, in

moldy ensilage, etc. As shown by Thom and Currie,[29] the mold of Roquefort cheese will grow in places at oxygen concentration much lower than that of the atmosphere, hence becomes dominant in cavities of cheese from which most other molds are automatically excluded by lack of oxygen.

Condensed milk. Sweetened condensed milk must be canned without the cooking process necessary to insure sterility. Certain sugar-tolerant yeasts grow slowly at the osmotic concentrations found (Hammer). Mold contaminations are not uncommon and occasionally develop as visible colonies upon the milk in the can. Such colonies produce harder and often more or less discolored areas in the milk held together by mold mycelium and by the curdling and digestion due to secreted enzymes. These masses have been called in the trade, "buttons." These "buttons" can only be produced by molds tolerant of the high concentration of sugar as found in this product,[25] such as members of the *Aspergillus glaucus, A. niger, A. versicolor,* and, perhaps *A. ochraceus* groups. Yeasts and molds are excluded from other condensed products by sterilization.

Dried milk; other desiccated products. Dairy products in powdered form are not subject to mold deterioration as long as they are properly protected from the local absorption of moisture. They are not, however, sterile, since mold spores and yeasts are often present and capable of growth upon any product into which the materials enter without adequate cooking.

A Key to Aid in the Identification of Molds Commonly Found in the Dairy and upon Dairy Products

Section I is to be used in connection with an examination of the moldy products by the hand lens (magnification 5 to 10 times). The information thus gained may be confirmed and extended by the use of Section II in connection with the study of the molds in culture by the aid of the compound microscope.

In this key each number ordinarily appears twice, once for each of a pair of contrasted characters. Occasionally a number is used but once, to repeat and extend a caption which applies to a series of numbers directly following. Less often, a single number is applied to three or more members of a related group.

SECTION I.

1. Mold species commonly found upon dairy products in general.............. 2
1. Mold species recognizable upon special products.......................... 7
2. Species forming a wrinkled, almost colorless membrane at the surface of liquid, semi-liquid or soft products, or upon the surface of wet utensils
Oidium lactis. See also 12 and 23
2. Species showing considerable aerial growth as fruiting stalks or felts of mycelium 3
3. Superficial growth a cottony tangled mass tardily showing fruit as indefinite powdery masses not separable into their elements with aid of the hand lens ....Fusarium sp. See 29
3. Superficial growth showing readily recognizable fruiting elements........... 4
4. Fruiting stalks terminated by "heads" of spores........................... 5
4. Fruiting stalks terminated by other masses of spores...................... 6

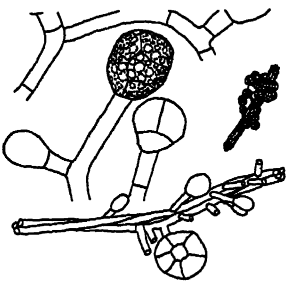
5. Heads when fully developed becoming either columnar masses or variously splitting to show more or less radiating chains or columns of spores The Aspergilli. See 39
5. Heads when undisturbed regular in outline, surrounded by a membrane (peridium) either smooth or fairly regularly marked.....The Mucors. See 38
6. Fruiting masses in blue-green, or yellowish green colors Species in Penicillium, Cladosporium, or Trichoderma. See 43, 31 and 49
6. Fruiting masses in yellow, olive-yellow, brown to black Species in Alternaria, Homodendrum (Cladosporium), Epicoccum, or in the Penicillium (Scopulariopsis) brevicaule group..............See 25, 32 and 46a
7. Molds of butter 8
7. Molds of other products 10
8. Butter showing colored mold colonies.................................. 9
8. Butter showing deterioration without visible mold or only very slight whitish powdery surface....................................Oidium lactis. See 23
9. Butter showing superficial masses of green or blue-green mold spores; various species often P. roqueforti.............................Penicillium. See 43
9. Butter showing submerged green or greenish brown growth, commonly Cladosporium. See 31. Less commonly Penicillium. See 43
10. Molds of cheese.. 11
10. Molds of other dairy products.. 17
11. Molds in the interior of cheese.. 12
11. Molds growing on the surface of cheese................................. 13
12. Green mold in the air spaces or cracks in Roquefort, Gorgonzola, Stilton and related forms and in deep narrow cracks of Cheddar P. roqueforti and related varieties
12. Uncolored growth with spores largely submerged in the cheese.....Oidium lactis
13. Any of the species already cited may be found on the rind of cheese held under conditions favoring mold growth.
13. Upon particular cheeses the following forms are significant................. 15
15. Mold forming dark brown discolored spots in the rind of Swiss cheese, with more or less gray-green to brown spores on the surface. Penicillium casei Staub
15. Molds of Camembert and related cheeses................................ 16
16. Cottony bluish gray to gray mold causing ripening or cottony white to cream color mold, otherwise identical in appearance P. camemberti Thom. P. caseicolum Bainier. See 45
16. Avellaneous or pale brown, or even cream-colored powdery mold colonies upon old cheese and imparting an ammoniacal odor P. brevicaule and varieties. See 46
17. Mold colonies recognizable with the hand lens do not develop upon properly handled milk powders, evaporated or condensed milks; when mold is found upon these products they should be handled by culture methods..See Section II

SECTION II.

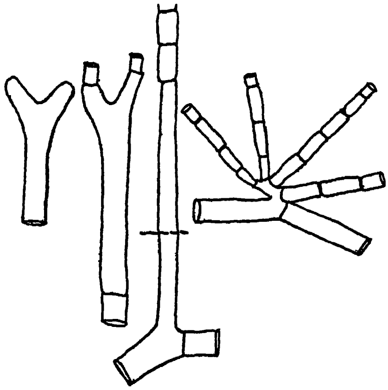
Molds in Culture from Dairy Products.

18. Species forming compact colonies suggesting those of bacteria consisting of individual organisms or irregular clusters of budding individuals but without definite hyphal networks or mycelia in ordinary culture or upon dairy products ....Yeasts (Saccharomycetes)
18. Organisms forming hyphal networks bearing spores or conidia upon more or less differentiated fruiting hyphae....................................... 19
19. Hyphae very delicate, 1μ or less in diameter, growing very slowly and forming close-textured tubercular masses on and under the surface of culture media, tardily producing white or bright-colored cushions of very short hyphae and tangled delicate chains or minute spores; characteristic and penetrating odor ....Actinomycetes
19. Hyphae coarser; colonies more broadly growing with larger and coarser forms of spores. (Molds as more generally understood)................. 20
20. Conidia-bearing filament (conidiophore) essentially as unbranched hypha with a conidia-bearing apex 21
20. Conidiophore showing special conidia-bearing structures at the apex, or developing characteristic branching systems................................ 34
21. Conidia borne singly on the stalk...................................... 22

21. Conidia borne in some type of chain or aggregate.......................... 23
22. Conidia forming terminal but multicellular balls or clubs
Epicoccum sp. Macrosporium sp.
23. Conidia developed as persistently cylindrical segments cut off successively from the apex of conidiophores and remaining as unbranched chains.
See Oidium lactis and associated forms



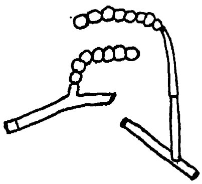
Epicoccum



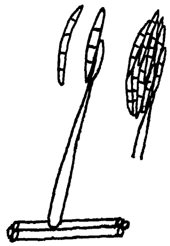
Oidium lactis

23. Conidia assuming some characteristic shape or arrangement................. 24
24. Conidia persistently in chains.. 25
24. Conidia assuming some other aggregation.................................... 32
25. Conidia unicellular as met in cultures.. 26
25. Conidia becoming clavate or oblong, several celled masses............Alternaria
26. Conidial chains remaining unbranched. In these species the new cells are at the base of the chain, the ripe ones at the apex.......................... 27
26. Conidial chains commonly branched.. 30
27. Conidia becoming globose 28

Alternaria



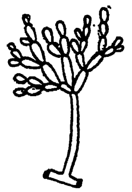
Catenularia



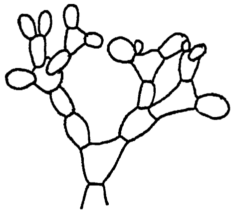
Fusarium

27. Conidia some other shape.. 29
28. Colonies small, firm, close-woven masses of rather delicate mycelium producing very short crowded conidiophores and chains of brown spores 2 to 3μ in diameter ....Catenularia
28. Various species with powdery fruiting areas in varying colors, white, yellow, orange, red, etc., have been described as species of Oidium, Oospora, Monilia, etc.
29. Conidia becoming fusiform elliptical; colonies showing long chains of fusiform elliptical "microspores" appear frequently from the presence of species of
Fusarium
29. Other colonies with similar spores have been described. Usages and descriptions are very indefinite................Monilia sp. Oidium sp. Oospora sp.

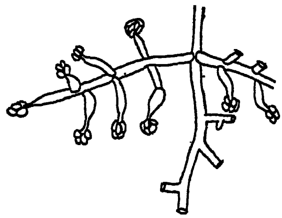
30. Conidial chains becoming branched and more or less bush-like masses by the repeated budding of new conidia from the tips of the cells already formed. In these forms the new cells are at the tips of the branches............... 31
31. Colonies delicate white or tardily blackening in submerged areas. Sporotrichum sp.
31. Colonies gray or olive, to brown or almost black. Cladosporium and Homodendrum
31. Colonies becoming heaped with powdery orange masses of spores. Monilia sitophila



Cladosporium



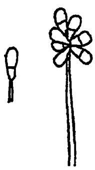
Monilia sitophilia



Trichoderma sp.

32. Conidia becoming massed together but without accessory structures.......... 33
32. Conidia forming a head with accessory structures.......................... 35
33. Conidia 1-celled, becoming massed in globules in more or less mucilaginous materials........................Cephalosporium and several related genera
33. Conidia several-celled, usually sickle-shaped and mostly arranged more or less in parallel packets ....Fusarium

Cephalothecium



Trichothecium

34. Conidiophore developing accessory conidia-bearing branches or structures at the apex 35
35. Conidia 2-celled, in a zig-zag series produced by proliferation of the apex of the stalk or conidiophore; colonies salmon to rosy.......Trichothecium roseum
35. Conidia 1-celled; conidial apparatus more complex......................... 36
36. Conidial apparatus a head or definite terminal structure.................... 37
36. Conidial apparatus a characteristic branching system....................... 40
37. Head a capsule, spores produced inside the head.......................... 38
37. Head with a central vesicle and radiating sterigmata with spores borne outside the mass in chains.. 39
38. Conidial head a capsule or sporangium with the conidia developed within an outer wall or peridium; mycelia hyphae unseptate; showing abundant protoplasmic movements during the growing period; usually producing much aerial mycelium with mostly globose heads upon more or less differentiated branches. A great group with many common forms, separable only with great difficulty and requiring special literature........ The Mucors, Rhizopus, etc. Three of the common series as encountered constantly in dairy culture are separated as follows:
38a. Heads or sporangia terminal on stocky sporangiophores arising separately from the substratum.......................................Mucor mucedo
38b. Terminal heads followed by the development of a series of smaller heads on divergent branches below...........................Mucor racemosus group
38c. Black heads borne upon stocky sporangiophores produced in clusters from a common base on long stolons attached by rhizoids to the substratum Rhizopus nigricans

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PART IV

THE NUTRITIONAL VALUE OF MILK AND MILK PRODUCTS. THE PHYSIOLOGY OF MILK SECRETION

Chapter XIV

Nutritional Value of Milk and Milk Products

Nutritional Requirements of Mammals

An animal consumes food for two main purposes. It needs, in the first place, a supply of energy as a basis for the activities which are the manifestations of its life. Every muscular movement carried out by animals is accompanied by the release of energy, and this energy is supplied by the chemical changes undergone by the food within the body. In addition to the energy required for muscular movement, mammals need energy to supply the heat which is necessary to keep their bodies at a constant temperature higher than that of the surroundings, and this energy also is supplied by the chemical changes undergone by their food.

Energy, however, is not the only factor supplied to animals by their food. In addition to energy, they must have certain particular compounds in their food which are used for various purposes. The requirement for particular compounds varies greatly according to the age and condition of the animal, and will be discussed more fully later. For the present it is sufficient to point out that a young animal is constantly adding to the material of its body, and that these additions can be built only from certain particular kinds of food.

Nutritive energy required by mammals. The food compounds from which animals derive energy are the proteins, fats, and carbohydrates. These are converted within the animal body to urea and other nitrogenous extractives, carbon dioxide, and water. The quantities of energy given out by these chemical changes undergone by the food within the body can be calculated, and from a knowledge of the chemical nature of the food it can be predicted just how much energy a given quantity of it is capable of yielding to the animal body. All forms of energy can be readily converted into heat, and energy is therefore usually measured in terms of heat. The unit of energy most commonly used in physiological work is the large Calorie, which is defined as the quantity of heat required to raise the temperature of a kilogram of water from 15° to 16° C.[190]

Many experiments have been carried out to determine the quantities of energy which the three great classes of foodstuffs will yield to the animal body; and it has been found that a gram of protein will yield, on the average, 4.1 Calories; a gram of fat, 9.3 Calories; and a gram of carbohydrate, 4.1 Calories.[222] From these figures the nutritive energy of any given food whose protein, fat, and carbohydrate contents are known can be calculated. The energy per unit of weight of the food calculated

in this way has been called its "metabolizable energy," and this term will be used in the further discussion of this subject.

If the quantity of energy which an animal needs to carry on its daily life is known, then the amount of any given food which it needs to supply that energy can be calculated from the metabolizable energy of the food. Much experimental work has been done to determine the energy requirements of human beings and of various other animals, and it has been found that they vary with the size, age, and activity of the animal, with the external temperature to which it is exposed, and with other factors. In comparing animals of different sizes, it has been found that the energy requirement is fairly closely proportional to the body surface, and the energy requirement is therefore often given per unit of body surface.

A full discussion of the energy requirements of men and of animals is given in Lusk's text-book on the subject.[172] Here it will be possible to give only an outline of the requirements of human beings.

The subject has been studied by determining the heat output of human beings resting in bed at a temperature of about 24° twelve hours or more after they have had their last meal. The heat output under these circumstances may be regarded as close to the minimum of which the body is capable and is called the "basal metabolism." The basal metabolism of normal men is about 950 Calories per square meter of body surface per 24 hours. This means that the average man weighing about 65 kilograms would have a total basal metabolism of about 1,700 Calories in 24 hours. Women have a basal metabolism about 10 per cent less than that of men per square meter of body surface, while children have a higher basal metabolism.

The heat given out by a man resting in bed twelve hours after he has had his last meal comes from the oxidation of his own body substance. If he is kept resting in bed and given food just sufficient to supply the Calories of his basal metabolism, the oxidation of his body tissues will be very much reduced; most of the heat output will then come from the oxidation of the food consumed. But there will still be some heat coming from the oxidation of body tissue under these circumstances, because food consumption always causes more heat output than occurs under fasting conditions. The tendency of different foods to stimulate the output of animal heat has been carefully studied, and it has been found that carbohydrate stimulates heat output equal to about 6 per cent of its metabolizable energy; fat, about 13 per cent; and protein, between 35 and 40 per cent. These different tendencies of different foods to stimulate the output of animal heat are called the "specific dynamic actions" of the foods.

In order to prevent entirely the oxidation of body substance in a man resting in bed, enough metabolizable energy would have to be given in the food to cover not only the basal metabolism of the subject, but also the specific dynamic action of the food. On account of the different specific dynamic actions of the three great classes of foods, more metabolizable energy would be required in the form of fat than carbohydrate, and more in the form of protein than of either fat or carbohydrate.

For human beings under normal conditions more food is, of course, required than under conditions of complete rest at a temperature of 24°, the amount being determined by the activity of the individual under consideration and by the external temperature. The amounts of energy required for different kinds of work have been determined by studying the food intake of human beings engaged in such work. The requirements vary from about 2,500 Calories daily for tailors up to 8,000 Calories for lumbermen.

An idea of the amounts of food which would supply the energy needed under various circumstances can be gained from a knowledge of the metabolizable energy contained in staple foods such as milk and bread. A liter of milk supplies about 700 Calories, and a kilogram of bread about 2,600 Calories. The daily requirement of an average man doing sedentary work would therefore be covered by about 3½ liters of milk or by 1 kilogram of bread, while a lumberman would require about three times these quantities. The ordinary loaf of bread weighs about half a kilogram.

Chemical elements and compounds required for the nutrition of mammals. As appears from the discussion which has just been given, the question of the energy requirements of animals is approaching a quantitative solution. That of their chemical requirements, on the other hand, is in a much less advanced state. We are still far from knowing, in a qualitative way, all of the chemical compounds which are required for nutrition, and the question of the quantities of these numerous compounds which are required for different animals under different conditions has hardly been touched. Still, great advances have recently been made in our knowledge of the chemistry of the animal body and of foodstuffs, and these advances are beginning to find an application in the practice of nutrition.

Factors which determine nutritional requirements. As has been pointed out in the previous section, the nutritional requirements of animals vary. The factors which cause variation in their energy requirements have already been considered, and it will be convenient to take up the factors which cause variation in their chemical requirements at this point.

An adult animal under maintenance conditions requires food chiefly as a supply of energy. If, instead of being kept under maintenance conditions, it is obliged to do work as a draft animal, or if it is fattened, its food requirements will be increased, but the increased demand will be chiefly for energy. Either muscular work or fat can be produced from any kind of fuel food. The case is quite different, however, if the animal is female and is caused to produce young. Young animals contain chemical elements and compounds which can not be produced from every kind of food, and the female animal which is carrying young must either get these elements for them from her food or take them from her own body. The case of a growing animal of either sex is like that of the reproducing female; growing animals must have a number of particular chemical compounds in their food to provide for the material which is constantly being added to their bodies. Finally, animals which are producing either milk

or eggs are in the same category as growing animals and females which are carrying young. Milk and eggs contain nearly all of the chemical elements and compounds which are necessary for the growth of young animals, and the milking or egg-producing female can get these elements and compounds only from certain particular foods.

Our present day knowledge of the chemical elements and compounds required by animals in their food has come to some extent from a study of the chemistry of foods, of the animal body itself, and of such products as milk and eggs. It is obvious that an animal which is growing must get at least as much of each chemical element from its food as appears in the new body substance. But this truth advances us only a very little way toward a solution of the practical problems of nutrition. Numerous experiments have demonstrated two additional important propositions. In the first place, animals have only a limited capability of converting one chemical compound into another; the fact that a food contains all the chemical elements contained in the animal body is no guarantee that the animal will be capable of building its body substance from that particular food. In the second place, there is always considerable wastage in nutrition, and, for the making of a given quantity of body substance, animals always need more of each element and compound in their food than appears in the finished body material. The study of the chemistry of the animal body, of animal products, and of food, therefore, must be supplemented by a study of the chemical transformations which occur within the living animal and of the unavoidable wastage of nutrition.

In making a general survey of the subject, it must be remembered also that animals and men have managed to nourish themselves for many ages without any knowledge of chemistry, and that even our present day knowledge of the chemistry of foods and other organic materials is very incomplete. Advances in the science of nutrition have very commonly been made as the result of practical observation. It is usually first found that animals fail to grow or develop some pathological condition on certain rations, and the attempt is then made to explain the observation from the chemistry of the ration in question as related to that of the animal body—not always with complete success. In any discussion of the chemistry of nutrition, therefore, it is necessary to keep clearly in mind the fact that we are dealing with a subject which is only partially known.

Place of protein, fat, and carbohydrate in nutrition. It is an old observation that the animal body consists largely of protein, fat, and carbohydrate. Foods also contain these three classes of compounds, and it is natural to suppose that the body protein is made from food protein; body fat from food fat; and body carbohydrate from food carbohydrate. Protein, however, has a more complicated chemical structure than either fat or carbohydrate. Whereas the two latter classes of chemical compounds contain only carbon, hydrogen, and oxygen, the first named contains these three elements, and, in addition, nitrogen and sulfur, and sometimes phosphorus. It is obvious, therefore, that, while either body fat or body carbohydrate might be made from food protein by the living

animal, body protein could not be made from either food fat or food carbohydrate.

Numerous experiments have shown that the living animal body actually has the power of making carbohydrate and fat from the protein contained in food. Protein, therefore, takes a predominant position among the three great classes of organic foodstuffs; it must be present in considerable amounts in the food of any animal which is either growing or yielding a protein-containing product. As it can be used for fuel in the animal body, as well as for body building, the question may be asked whether carbohydrate and fat need be present in the food at all. The question whether fat is a necessary constituent of the diet is complicated by the fact, which will be discussed more fully later, that animals can not thrive unless their diet contains small amounts of several substances whose chemical nature is not yet fully known. Some of these substances are either chemically related to the fats,[5e] or, like vitamins A and D and E, are associated with them in nature. It may therefore be said that animals can not thrive on diets which are strictly free from all kinds of fat.

Experimental work has shown, however, that animals can live in good health for fairly long periods on diets which contain very little of either carbohydrate or fat. This work is very interesting from the practical point of view in showing that protein can be substituted, to a large extent, for fat and carbohydrate in the diet, and that either of the two latter substances can be substituted for the other. It should not, however, be taken to mean that it makes no difference to any kind of animal how little fat and carbohydrate it gets in its food if only it gets enough protein. On account of the high specific dynamic action of protein, diets made up largely of this substance will cause animals to waste more heat than they otherwise would. Such diets will therefore be uneconomical at all times, and decidedly disadvantageous in warm weather when animals have difficulty in getting rid even of their minimum heat production. Further, the experiments in which carbohydrate and fat have been reduced to a minimum in the diet have been carried out on only a few species of animals, and it is far from certain that the results obtained with these would apply to all.

Relation between the chemical nature of protein and its value in nutrition. The chemical nature of protein has been taken up in earlier chapters of this book, and it is only necessary here to point out in a general way the bearing of its chemical nature on nutritional questions.

It must be remembered, in the first place, that the protein molecule is composed of amino acids and that the proteins are broken up during digestion into single amino acids and short-chain polypeptids; this has brought up the question whether mixtures of all the amino acids known to be present in proteins could not take the place of proteins in the diet. Experiments in which animals have been nourished on mixtures of single amino acids have met with considerable success. This work has been reviewed by Rose.[221b] He and co-workers [221b, 68a, 280a] report experiments of their own in which a mixture of nineteen carefully purified amino acids

was fed to rats to supply the protein requirements. This mixture included "all the well recognized amino acids except hydroxyglutamic acid." The rats failed to grow. The addition of a source of hydroxyglutamic acid to the diet had no effect; but, when small amounts of gelatin or gliadin or especially of casein or fibrin [221c] were substituted for a portion of the amino acid mixture, the rats grew. Even hydrolyzed casein was effective as a supplement to this synthetic mixture of amino acids, the effective material being associated with the monoamino-monocarboxylic acid fraction of this hydrolysate. Rose [221c] states that this material is unquestionably an amino acid, and believes that it has a relatively simple chemical structure. It appears, therefore, that when all of the amino acids occurring in proteins become known and are available for experimental work, it may be found that animals may be normally nourished on synthetic mixtures of them as the only dietary supply of their protein requirements. Work is still in progress looking to this end.

Another question to be answered, therefore, is whether all of the amino acids found in proteins are necessary in nutrition, or whether certain ones can be made by the animal from certain others. It has already been pointed out that not all proteins contain all the known amino acids. Proteins which are deficient in one or more of the known amino acids are generally spoken of as incomplete. Growing animals have been fed on various incomplete proteins, and in certain instances it has been found that their growth soon ceased under such circumstances. These results point to the belief that some, at least, of the individual amino acids are necessary for the nutrition of growing animals—that animals do not have an unlimited capacity for synthesizing amino acids or for producing one amino acid from another.

Many experiments have been carried out to throw light on the relative importance of the various amino acids in nutrition. Through what might be called a lucky metabolic accident, it has been shown that one of the amino acids, glycine, can be synthesized in the mammalian body, and the fact that the mammal can synthesize glycine brings up the question of whether it can synthesize other amino acids. This question has been studied in experiments in which animals have been fed on proteins deficient in certain amino acids (incomplete proteins), either with or without the addition of the missing amino acids, while their changes in weight and general well being have been determined. In other experiments various mixtures of single amino acids have been used. Reviews of the results of the older work in this field are given by Lusk,[172] and Van Slyke,[268] and by Plimmer.[212] This field is a complicated one and is still in a very early stage of development.

Experiments of this sort with incomplete proteins have demonstrated clearly that when they lack tryptophane, lysine or cystine they are inadequate for the normal nutrition of the rat and that some source of these amino acids must be supplied in the diet. More recently Jackson and Block [189b] have shown that another sulphur-containing amino acid, which has been discovered to occur in a number of proteins and is called methi-

onine, can take the place of cystine in the diet. Rose and Cox [221a] have demonstrated that histidine is also an indispensable component of the diet, and that it and arginine are not mutually interchangeable in metabolism as these amino acids were once thought to be. Womack and Rose [284a] have also reported experiments which "demonstrate that both leucine and isoleucine are indispensable dietary components."

On the other hand, glycine apparently is not the only amino acid which it is unnecessary to supply in the diet. Scull and Rose [228a] found that with the rat the increments in tissue arginine may be two to three times as large as may be accounted for by the arginine ingestion. They conclude that arginine, like glycine, may be synthesized in the organism of this animal, and that it is therefore not an indispensable component of its diet. St. Julian and Rose [224a] report feeding experiments with aspartic acid, the glutamic acids and the prolines which indicate that rats also require very little or none of these amino acids in their rations. With tyrosine and phenylalanine there appears to be a lack of agreement as to whether it is necessary or not to supply them in the diet. With all of the other amino acids there is very little or no evidence at hand that would bear upon this question.

In considering the essential nature of the amino acids that appear to be indispensable in the diet, the question naturally arises as to how specific this requirement actually is in each instance. Cox and Rose [41a] and Harrow and Sherwin [104a] have studied this problem very thoroughly in the case of histidine. They fed a number of closely related synthetic imidazole derivatives as substitutes for this amino acid. All were ineffective except those which might themselves be considered as occurring naturally in the animal organism as intermediate products in the metabolism of histidine itself—i.e. imidazole lactic acid [41a, 104a] and imidazole pyruvic acid.[104a] Imidazole acrylic acid was either ineffective [41a] or a very poor substitute [104a] for histidine. But as substitutes for lysine [177a] and tryptophane [189a] even *dl*-α-hydroxy-ϵ-aminocaproic acid and *l*-β-3-indole lactic acid, which bear respectively the same relation to these amino acids as imidazole lactic acid bears to histidine, are ineffective.

On the other hand, du Vigneaud, Dyer, and Harmon [269b] found that, besides methionine, the next higher symmetrical homologue of cystine, which du Vigneaud and Meyer [269a] had made from methionine and called homocystine, can likewise replace cystine in the diet. They regard the homocystine as possibly an intermediate product in the physiological conversion of methionine to cystine. It is regarded as entirely likely that alanine may be synthesized from pyruvic or lactic acids which occur in the organism as intermediate products of carbohydrate metabolism as well as of the metabolism of this amino acid.

Mineral requirements of mammals. The animal body regularly contains a number of chemical elements which are not necessary constituents of either protein, fat, or carbohydrate. These are the so-called mineral or ash constituents of the body; they consist largely of combinations of calcium, magnesium, sodium, potassium, and iron with phos-

phorus, chlorine, iodine, and fluorine. The phosphorus is commonly combined with the metallic elements as phosphate. For many years past, experiments have been carried out in which growing animals have been fed on rations deficient in one or more of these mineral elements, and it has always been found that growth ceases sooner or later. It is regarded at present as entirely established that an animal can build its body only from food which contains all the chemical elements that are regularly found in its body.

Natural foodstuffs commonly contain many of the mineral elements which are necessary for nutrition, and the question of the mineral requirements of animals resolves itself in practice into the question how far the various dietaries which are commonly used are likely to be deficient in one or another of these elements. The mineral requirements of farm animals have been much investigated lately, and it has been clearly shown that farm animals under practical conditions frequently suffer from receiving diets which are deficient in certain mineral elements. The mineral requirements of human beings have been less closely investigated than those of farm animals on account of various difficulties which stand in the way of such investigation, but enough work has been done in this field to show that mineral deficiencies in the diet of human beings are not uncommon.

The vitamins which animals require. Recent work in nutrition has been largely directed toward determining how far our present knowledge of chemistry and of the chemical transformations which are possible within the animal body make it possible to predict whether an animal will grow on a ration with a given chemical make-up. Numerous experiments have shown that it is not possible to get satisfactory growth on a diet which contains all of the chemical elements and compounds, known to be necessary for nutrition, in purified form. Small amounts of certain natural food substances must be added to chemically purified rations in order to cause animals to grow satisfactorily. The conclusion has been drawn that the natural foods contain a number of organic compounds, either recently discovered or still unknown, which are necessary in nutrition, and these dietary essentials have been called "vitamins." A number of such vitamins is now fairly well recognized as being present in various foods, and will be fully discussed later.

It will be seen from the foregoing that the question of providing in the food the raw materials for the manufacture of the body substance of animals, or of such animal products as milk or eggs, is complicated and still largely unexplored. It may be said that the growing or milking animal can manufacture such and such compounds from such and such others, while there are certain elements and compounds which it must have in its food. Minimum requirements may be set for all the known essential elements and compounds by saying that there must be at least as much of each in the daily ration as is to be put into the daily body growth or milk or egg yield. In certain cases fairly good approximations can be made as to the amount of compound in the diet which will be required for a given output in growth or milk or egg yield. Beyond this, however, it

must be said that not all of the chemical compounds which must be present in the food in order to provide for growth are known and that, of those known, there are many in regard to which only the roughest guess at the quantitative requirements can be made.

Nutritional requirements of adult mammals which are not reproducing. In the foregoing discussion, the chemical requirements for growth, for reproduction, and for milk secretion have been considered, and there has been comparatively little discussion of the chemical elements and compounds required in the diet of adult animals which are not either reproducing or secreting milk. It is entirely reasonable to suppose that such animals will be able to get along with much smaller quantities of many chemical elements and compounds than are required by growing, reproducing, and milking animals; and experiments have abundantly demonstrated that this is the case. But it would be a great mistake to suppose that adult animals require only a supply of energy in their food and that their other requirements can be neglected.

As contradicting such a supposition, it may be pointed out that adult human beings develop scurvy when deprived of fresh meat, fruit, and vegetables, and that they develop beri-beri when fed largely on highly milled cereals. The effects of other deficiencies in the food of non-reproducing adults have not been brought into such prominence, but it seems probable that they also would be followed by bad effects of one kind or another.

Many experiments have shown clearly that adult animals require protein and mineral elements in their food very much as do growing animals, except that the quantities required are not so large.

When adult animals are starved, they continue to excrete feces and urine. The feces contain protein and mineral elements, and the urine contains the nitrogenous catabolic products of protein metabolism and all of the mineral elements of which the body is composed. As starvation progresses, the loss of these materials from the body becomes very small, but it does not stop altogether, and it is justifiable to suppose that the amounts of the various elements and compounds lost from the body in starvation represent roughly the amounts of those elements and compounds which must be supplied in the food in order to keep the body's stock of each particular constituent up to normal.

In the case of protein, experiments have been carried out to determine whether under any circumstances the loss of this substance from the body can be reduced below the starvation minimum, and how the body can be prevented from losing it. Animals have been fed on rations of carbohydrate and of fat without protein but with sufficient energy to supply their requirements, and their daily loss of nitrogen has been determined when they were on these rations alone and on the same rations plus various amounts of different kinds of protein.

It has been found that a diet of fat without either protein or carbohydrate will not reduce the loss of nitrogen from the body below that which occurs in starvation,[172b] while a diet of carbohydrate alone or a

mixed diet of carbohydrate and fat will reduce it very considerably. The feeding of carbohydrate without protein will not, however, abolish the loss of nitrogen from the body and animals fed on such rations ultimately die.[172c]

It is an interesting question whether adult animals require all of the amino acids which are needed for growth and reproduction; the following experimental material bears on this question. Gelatin is an incomplete protein, lacking, in addition to others, the three amino acids, tyrosine, cystine, and tryptophane. When an adult animal is supplied with the nutritive energy which it requires in the form of gelatin, it continues to lose large amounts of nitrogen from its body, but it may be brought almost into nitrogen equilibrium by the addition of the three missing amino acids.[172a]

There is much experimental evidence, therefore, which indicates that the chemical requirements of adult animals are qualitatively the same as those of growing and reproducing animals. The former have been shown to require for their well-being many of the vitamins, minerals, and amino acids which are needed by the latter. The discussion should not be closed, however, without referring to some very interesting experiments which throw further light on the subject. In these experiments young rats and mice have been fed on certain incomplete proteins with and without the addition of various single amino acids. The diets also contained in all cases fat and carbohydrate and the various minerals and vitamins which are necessary for the nutrition of the animals under consideration.

Zein is a protein obtained from maize, from which lysine and tryptophane are entirely absent. When young rats and mice are fed on diets in which zein is practically the sole source of protein, they decline in weight and die in a short time. Lysine added alone to such rations does not prevent the decline. Tryptophane alone, however, does prevent the decline, for some time at least, and keeps the animals alive for a much longer time. When tryptophane and lysine are added together in proper quantities normal growth ensues.

Further results which supplement these have been obtained by substituting gliadin for zein in the diet. Gliadin is a protein obtained from wheat which contains all of the amino acids necessary for nutrition, though only very small quantities of lysine, arginine, and histidine. Young rats have been kept alive for a year and a half on diets in which gliadin was the sole source of protein. During the whole of this period they remained in good health and failed to grow, but resumed their growth when a more adequate protein was substituted for the gliadin. For an account of these experiments with references to the original articles, see Lusk.[172e]

These experiments illustrate beautifully the differences in the nutritive requirements of young and adult animals, and they suggest very interesting differences in the importance of the different single amino acids. It would appear that animals can not live at all without tryptophane whereas they can live for at least long periods with very little

lysine. They can not, however, build body tissue without an adequate supply of the latter amino acid. Diets quite deficient in it, therefore, produce in young animals the very noticeable and remarkable phenomenon of a stoppage of growth, whereas they may have no visible effect on adult animals.

The results do not, however, indicate that young animals differ from adult animals in regard to the compounds which they require in their food in order to maintain life. On the contrary, they indicate that there is no necessary connection between growth and life in young animals, and seem, if anything, to strengthen the view that the nutritive requirements for the maintenance of life are the same in adult as in young animals.

Some evidence indicating that the nutritive requirements of young mammals may be qualitatively different from those of adults is to be found in the attempts which have been made to nourish young mammals without milk. It has never been possible to do this satisfactorily with most species, and one is justified in at least suspecting that milk may contain chemical compounds which are not present in other foods and are more necessary for the young than for the adult of the species. But such compounds have not yet been demonstrated. The experimental evidence bearing on this subject will be considered in another place.

Initial Considerations Regarding the Nutritive Properties of Milk

In beginning the consideration of experimental work bearing on the nutritive properties of milk, it is worth while to keep certain important general aspects of the situation in mind, namely, the natural food habits of mammals and certain changes which take place in the mammary secretion at the beginning of lactation.

In the case of many mammals the young are nourished entirely on milk for the first weeks or months of their existence. They are then forced gradually to substitute other foods for the milk, and usually live through a considerable part of the period of growth and all of adult life without consuming any milk or milk products at all. Simple observation shows, therefore, that the mother's milk is a satisfactory food for young mammals, and that older mammals can thrive very well without it. But, from the human point of view, it is often advantageous to nourish young mammals with the milk of foreign species or with as little milk as possible, and to use milk and milk products as food for adult animals. This has led to the very extensive experimentation which has been carried out on the nutritive properties of milk. A great deal of light is thrown on many of the experimental results by certain recently acquired knowledge regarding the initial mammary secretion,—colostrum,—and this part of the subject will be immediately considered.

Colostrum and Its Importance in Nutrition

Colostrum is a fluid secreted by the mammary gland for a short period after parturition. It is quite different from normal milk in composition and, since it is secreted universally by mammals, its appearance can scarcely be regarded as a fortuitous circumstance without particular significance. Yet as late as 1922 the importance of colostrum was not especially stressed in text books and its precise function remained more or less in doubt. As Traum [264] observes it was "generally taught that colostrum acts as a laxative, and that it is nature's provision for aiding the elimination of deleterious gastro-intestinal contents of the newborn." The early work of Howe [128] indicated that colostrum was not exactly laxative although its ingestion did not delay the excretion of fecal material as did milk feeding to the newborn.

Composition of colostrum. Colostrum is essentially an extremely rich solution of globulin in a fluid which otherwise strongly resembles milk. Colostral globulin may run as high as 13 per cent, is identical with serum globulin and is the only milk protein that is identical with any blood protein. This was proven by Crowther and Raistrick [48] and later confirmed by Wells and Osborne [278] using the anaphylactic test. Colostrum contains somewhat more casein, albumin, chlorine and total ash than normal milk and somewhat less lactose. The casein, albumin, fats and lactose are the typical milk constituents not found elsewhere in the body, and the serum albumin and fibrinogen of the blood do not appear in the colostral secretion.

The colostral globulin content sometimes exceeds that of the blood. It has usually been regarded as a true secretion, though some authors have regarded it as a mere leakage from the blood vessels. Woodman and Hammond [286] have taken this latter point of view. Their article contains references from which the subject can be looked up in detail.

Crowther and Raistrick [48] established the identity of the caseinogen, lactoglobulin (globulin) and lactalbumin from colostrum and from milk, and also demonstrated the presence of about 0.03 per cent globulin in normal milk. The comprehensive work of Engel and Bode [81] went far to show that colostral fat early displays the distinctive characteristics of milk fat.

Table XCVII has been taken from a recent article by Engel and Schlag.[80] This work was selected because it was recent, careful, and comprehensive, and shows the typical variations in colostral constituents over a characteristic colostral period. More constituents and physical constants were determined than by most investigators and samples were taken at shorter intervals than usual. In Table XCVII, columns containing constituents which vary in a regular manner have been placed first. The figures represent the results for one cow only. Engel and Schlag give similar tables for two other cows, which show essentially similar phenomena, though with minor variations.

Like many other investigators Engel and Schlag have included both

Table XCVII.—Figures showing the progressive change of colostrum into milk. (From Engel and Schlag.)

Time after calving	Acidity*	Specific gravity	Chlorides	Total protein (N × 6.37)	Casein	Albumin †	Ash	Dry matter	Coagulates on boiling	Sugar	Fat	Freezing point
			per cent	per cent	per cent	per cent	per cent	per cent		per cent	per cent	°C
At once	18.4	1.0670	0.1525	17.57	5.08	11.34	1.01	26.99	Yes	2.19	5.10	—0.605
6 hours	14.4	1.0437	0.1631	10.00	3.51	6.30	0.91	20.46	Yes	2.71	6.85	—0.555
12 hours	11.2	1.0368	0.1560	6.05	3.00	2.96	0.89	14.53	Yes	3.71	3.80	—0.566
24 hours	10.8	1.0343	0.1560	4.52	2.76	1.48	0.86	12.77	Yes	3.98	3.40	—0.575
30 hours	9.8	1.0318	0.1524	4.01	2.56	1.20	0.83	13.63	Yes	4.27	4.90	—0.570
36 hours	10.0	1.0320	0.1595	3.98	2.77	1.03	0.84	12.22	Yes	3.97	3.55	—0.570
48 hours	9.6	1.0319	0.1489	3.74	2.63	0.99	0.83	11.46	Yes	3.97	2.80	—0.580
72 hours	10.0	1.0331	0.1365	3.86	2.70	0.97	0.84	11.86	No	4.37	3.10	—0.575
96 hours	9.2	1.0335	0.1347	3.76	2.68	0.82	0.83	11.85	No	4.72	2.80	—0.555
5 days	8.5	1.0334	0.1312	3.86	2.68	0.87	0.85	12.67	No	4.76	3.75	—0.575
7 days	9.0	1.0320	0.1184	3.31	2.42	0.69	0.84	12.13	No	4.96	3.45	—0.570

* Acidity was determined by the Soxhlet-Henkel method which consists in titrating 25 cc. of milk with N/4 NaOH to a slight rose color with 2 cc. 2 per cent solution of phenolphthalein in alcohol as an indicator. One degree of acidity equals 1 cc. N/4 NaOH per 100 cc. milk.

† The figures for "albumin" really represent albumin and globulin.

the albumin and the globulin of colostrum under the term "albumin." In order to show the variations in globulin and albumin separately Table XCVIII is appended from the work of Crowther and Raistrick.[48] The cows were milked twice daily after parturition, the first milking evidently taking place some hours post partum.

Table XCVIII.—Globulin, albumin, and other nitrogen constituents contained in colostrum.

Milking	Total N	Casein N	Albumin N	Globulin N	Non-protein N
	per cent	per cent	per cent	per cent	per cent
1	2.40	0.75	0.14	1.32	0.19
2	2.01	0.68	0.17	1.02	0.14
3	1.44	0.59	0.14	0.59	0.12
4	0.97	0.51	0.11	0.31	0.04
5	0.76	0.46	0.07	0.20	0.03
6	0.75	0.46	0.06	0.20	0.03
7	0.69	0.42	0.06	0.18	0.03
8	0.65	0.46	0.05	0.12	0.02

From Table XCVII it will readily be seen that the secretion tends to become less acid during the colostral period, that the specific gravity, chlorine, total nitrogen, casein, "albumin," ash, and dry matter rather consistently fall, that the sugar content rises, that the fat tends to vary irregularly, certainly not in any characteristically distinctive manner; and that the freezing point remains fairly constant throughout. The milk ceases to coagulate with heat about the third day. The colostral period may extend through the first six to twelve days of lactation, depending upon the speed and completeness of the transformation. Usually the milk

after the sixth day is essentially normal. Weber,[277] however, holds that the full colostral period extends to twelve days. The most striking deviation of colostrum from milk is obviously its very high globulin content.

Function of colostrum. Turning to the function of colostrum we find that Hohlfed [128] demonstrated that dogs, goats and guinea pigs could be raised without colostrum, but he thought that growth in early life was more rapid when colostrum was fed. Bauer [11] showed that certain proteins and antigens which are present in maternal blood serum appear also in colostrum but not in milk. He suggests that, while the constituents of milk are produced exclusively in the mammary gland, some of those of colostrum come directly from the maternal blood, and that this circumstance may be of considerable importance for the welfare of the offspring during the early part of its extra-uterine existence.

Birk [22] observed that infants fed colostrum maintained a positive nitrogen balance and showed good phosphorus, potassium and magnesium retention, while controls fed milk showed negative balances.

Famulener was, however, the pioneer in outlining the most important function of colostrum. He [87] demonstrated that colostrum is the chief agent in bringing about the passive immunization of a suckling and that sucklings rapidly gain a well maintained antibody content in their blood by being fed colostrum. He immunized goats to sheep erythrocytes during gestation and found that the blood of newly-born kids, taken before they had suckled, showed no appreciable trace of hemolysins. Therefore little transfer of these bodies had taken place during gestation. But the colostrum of the mothers was found to be rich in hemolysins and the kids suckling this colostrum rapidly acquired a high blood content of these bodies. Older sucklings did not, however, absorb these hemolysins from the colostrum so quickly.

The amount of hemolysins in colostrum and in milk observed by Famulener corresponds perfectly with the curve for globulin content established by Crowther and Raistrick four years later; but as early as 1912 Famulener thought the two things associated. He concluded that "it seems of the highest importance that the newly-born child should, during the first days of its life, receive the colostral milk."

It was not until 1920, eight years after the publication of Famulener's work that Reymann [217] again studied the transfer of antibodies from mother to offspring in goats. He determined the transmission of bacterial agglutinins, which normally occur in the blood, as they passed from mother to offspring. In all cases he found that kids were born without agglutinins and that they obtained these from the colostrum in which agglutinins were always abundant. These antibodies disappeared both from the milk and from the kid's serum within a few days after parturition. Colon and typhoid agglutinins as well as the agglutinins against rabbit and horse blood corpuscles were studied. In the colostrum the antibody titer was higher than in the maternal serum, but agglutinins did not appear to be transmitted by the normal milk. The maximum antibody content might occur in the kid's serum eleven hours after birth.

Howell and Eby [134] confirmed the conclusions of these workers. Using rabbits they found that the antibodies sometimes disappeared almost entirely from the serum of immunized mothers after parturition. This was taken by Lewis and Wells [167] to indicate that antibodies were poured into the colostrum for the protection of the offspring, but the work of Howell and Eby did not specifically rule out the possibility of placental transfer.

Crowther and Raistrick [43] showed that globulin was the same in composition whether prepared from milk or from colostrum. By dialysis they resolved colostral globulin into two constituents, pseudoglobulin and euglobulin.

In 1918, Dudley and Woodman [61] published work on the chemistry of euglobulin and pseudoglobulin. They confirmed the work of earlier investigators in finding that these two compounds were very similar in appearance and properties and in the character of the amino acids which they yielded on hydrolysis, but found that they differed in phosphorus content. Somewhat later Woodman [285] announced the identity of globulin from cow or ox serum with that from colostrum; but he found that lactalbumin and serum albumin were very different substances. Some years before the time of the publications which have just been mentioned Porges and Spiro [214] had studied the globulin of blood serum and found that it consisted of three fractions: (1) A euglobulin, precipitated by 28 to 36 per cent of saturated sodium sulfate solution; (2) A pseudoglobulin I, precipitated by 32 to 42 per cent; and, (3) A pseudoglobulin II, precipitated by 40 to 46 per cent.

This work has all been examined because it contributes to a correct understanding of the important investigations started by Howe in 1921. Howe [129] first worked further on the precipitation of serum globulin with sodium sulfate, using both diluted and undiluted plasmas. He found definite zones of sodium sulfate concentration wherein he could precipitate euglobulin and pseudoglobulins I and II. He next [130] showed that the blood of a newborn calf contains no euglobulin or pseudoglobulin I but that after feeding the calf colostrum these globulins suddenly appear in its blood. If, however, the calf is fed only milk, euglobulin and pseudoglobulin I are absent from the blood for some time. But the feeding of colostrum always resulted in the rapid appearance of the missing globulin in calf's blood when ingested before twenty-one hours of age. Howe [131] then applied his sodium sulfate precipitation method to the determination of the proteins in colostrum and found there also euglobulin, pseudoglobulin I and pseudoglobulin II.

Howe's next investigation [132] showed that the high concentration of euglobulin and pseudoglobulin I which appears in the blood of a new-born calf just after it has had colostrum is transient. The concentration of these two proteins in the blood is greatly decreased after the first day or so even when the calf continues to receive colostrum, and it is not until the age of 12 or 14 months that the animal has again acquired the high concentration of them which is characteristic of adult life. Pseudo-

globulin II, on the other hand, is relatively constant in the blood from the time of birth on, whether the calf is fed colostrum or not.

Lewis and Wells [167] were inspired by Howe's work to make observations on the human subject. They repeated Howe's work, using the blood of human adults and young infants. They found that the blood serum of a new-born infant is virtually devoid of euglobulin, although it contains the same amounts of pseudoglobulin I and pseudoglobulin II as that of an adult. This would indicate that the human placenta, unlike that of the cow, is permeable to pseudoglobulin I. Lewis and Wells found also that euglobulin appeared and rose gradually in the blood of young infants even when they got no colostrum, but much more rapidly when they did get colostrum. The euglobulin fraction is associated with antibodies. Almost simultaneously with Lewis and Wells, Boyd [26] published her observation that the blood of new-born infants contains but small amounts of globulin, the euglobulin being negligible, but that this deficiency is rapidly compensated for by feeding colostrum. When colostrum is not fed, both the total globulin and the euglobulin fraction remain low in amount.

An account will now be given of other work contemporary with Howe's. Little and Orcutt [171] working with some of the same experimental animals used by Howe showed that *Bacillus abortus* agglutinins found in the blood of a new-born calf are obtained from the mother and are transmitted via colostrum. Unfed calves do not have these antibodies at birth, and their quantity is much lower in the blood if the calves are fed colostrum artificially than if they are permitted to suckle. They remain altogether absent from the blood if the calves are fed only milk.

Orcutt and Howe [201] next gave evidence associating the appearance of a definite protein factor in the blood of the new-born animal with simultaneous absorption of the agglutinins for *Bacillus abortus* from the mother through the colostrum. Withholding colostrum and feeding only milk resulted in the absence from the blood of the offspring of both agglutinins and globulins. The agglutinins could also be removed from serum or from colostrum by the same concentrations of sodium sulfate which precipitated the globulins. Moreover, after the ingestion of colostrum, globulins and agglutinins appeared in the calf's blood in quantities directly related to their concentration in the colostrum and to the amount thereof ingested. This gave considerable experimental foundation for the idea that this colostral phenomenon of passive immunization is non-specific and may be but a physiological reaction caused by the ingestion of certain proteins *per se*.

Certainly the work up to this point leads to the conclusion, in so far as calves are concerned, "that the formation of colostrum is for the purpose of presenting to the new-born animal a concentrated solution of serum globulin which carries antibodies from the maternal blood during the brief time these protective proteins can be absorbed unaltered from the alimentary canal in an active condition." [167]

In 1922 Smith and Little [258] attacked the problem of the importance

of colostrum for the new-born calf, and found that calves fed colostrum survived much more frequently than those that were deprived of it, even though the latter got plenty of milk. *Bacillus coli* was found all through the bodies of calves which had died as the result of not getting colostrum. Smith and Little conclude, like previous investigators, that colostrum protects the new-born calf against organisms which, later in life when the bodily protective agencies are more fully in operation, are harmless.

This was followed the next year by work [254] showing that calves at birth lack agglutinins and that colostrum is the most effective agent in transporting these antibodies from mother to offspring. *Bacillus abortus* was used and the prompt appearance of its agglutinins in a calf's serum after the ingestion of high titer colostrum was duplicated by feeding maternal cow serum in lieu of colostrum. Ragsdale and Brody [215] also found that calves frequently failed to survive when they were not fed colostrum and they further showed that the pasteurization of colostrum only slightly decreased its protective powers.

The work of Smith demonstrated that if colostrum, or its equivalent in cow serum, is not ingested by the young calf, the calf "lacks something which permits intestinal bacteria to invade the body and multiply in various organs. The rapidity and duration of this multiplication determines the fate of the calf. In most cases a rapidly fatal septicemia is the result. When resistance is greater, life may be prolonged or the animal survive indefinitely."

In 1924 Smith and Little [255] found that the feeding of colostrum was associated with albuminuria in young calves; feeding maternal serum acted similarly. Howe [138] then showed that the proteins excreted were euglobulin and the two pseudoglobulins, the same as those ingested with colostrum or blood serum. This appearance of euglobulin and pseudoglobulin in the blood and urine of a young calf was thus directly associated with feeding colostrum. The high nitrogen content of the feces of young calves was also associated with the colostrum fed.

The results so far given all tend to show that the ingestion of colostrum by new-born animals has a very important influence in protecting them against infection during the period immediately following their birth. But in order to complete the subject, it is necessary to report the work of Kuttner and Ratner [160] which indicates that the colostral antibodies do not have the same importance for human beings, and probably also for certain other animals, as they have for young calves. The work of these investigators is extensive and apparently well carried out. They first observe that previous work shows guinea pig, rabbit, and human placenta to be permeable to antibodies; but cow, goat, horse and sheep placenta impermeable. They then report their own experiments, which constitute strong evidence for the view that human infants commonly acquire their immunity to disease from the maternal blood directly through the placenta before they are born rather than from the colostrum just after birth. Kuttner and Ratner find that the blood of infants born from mothers who are not immune to diphtheria contains no antitoxin, while that of infants

from immune mothers contains just as much antitoxin as the mother's blood, even before the babies have received any colostrum. The colostrum of immune mothers, on the other hand, contains a much smaller concentration of antitoxin than does their infant's blood, and the antitoxin is not increased in the blood of such infants after they have received colostrum.

In addition to their work on the diphtheria antitoxin contained in the blood of mothers and infants, Kuttner and Ratner collected from certain New York hospital records eighteen cases of infants which had never received colostrum on account of the early deaths of their mothers. Two of these infants died from causes which were obviously not connected with their failure to receive colostrum. The rest survived for the periods that they remained in the hospitals, and the records do not indicate that they suffered in any way from not having received colostrum.

These authors conclude by drawing attention to the marked quantitative differences in the early mammary secretion of cows and human beings. The cow usually has 20 pounds of colostrum ready formed at parturition, while the human colostral secretion does not start until 12 hours after birth, and amounts to but 5 cc. in the first 24 hours, and to but 90 cc. in the first 48 hours.

The work of Kuttner and Ratner, taken in connection with that of Lewis and Wells and of other investigators, certainly gives strong reason for believing that colostrum is of much less vital importance to the human infant than to the young calf. But it would be rash to conclude from the work so far done that human colostrum has no importance at all as an immunizing agent. Diphtheria is only one of many forms of infection to which animals are liable, and it seems hardly safe to assume that all the others would behave just as diphtheria does. And in comparing the mortality of the series of infants studied by Kuttner and Ratner with that of the calves studied by Smith and others, it must be remembered that a new-born calf usually has much better opportunities for ingesting large quantities of bacteria than does a human infant under hospital management. Further, the infants studied by Kuttner and Ratner were not numerous enough, nor were their histories followed long enough to be conclusive in such a case. In several instances their histories were followed for a week or less, and the majority of them were not followed for as much as a month.

Very recent work has shown that colostrum, in addition to providing the new-born with specific antibodies, protects against disease in still another way. Dann finds that the vitamin A content of new-born animals is surprisingly low, and that there is a definite limit to the amount of vitamin A which can be passed from the mother to the offspring in milk.[48] But cow's colostrum often contains from 10 to 100 times as much vitamin A as the later milk from the same cow.[49] There is no evidence as yet as to whether or not the colostrum of human beings also has the function of transferring quickly from the mother to the new-born offspring a

large supply of vitamin A. The importance of this vitamin in protecting animals against infection will be discussed later in this chapter.

Further recent work on the importance of colostrum as an immunizing agent has consisted in an extension of principles already established to other forms of immunization and other species of animals. Debré, Ramon, and Thiroloix have shown that tetanus antitoxin passes through the human placenta from the maternal to the fetal blood, but does not appear in human colostrum.[52] Mason, Dalling, and Gordon have studied the placental and colostral transmission of immunizing bodies from mother to offspring, in sheep, cattle, horses, pigs, and dogs. They publish a table based partly on their own work and partly on that of others, which shows that in pigs and ruminants colostral transmission is of paramount importance, while placental transmission is of paramount importance in rodents, apes, and human beings. In carnivora the two paths of transmission are of more nearly equal importance.[183]

In work which will shortly be published, Earle, of the Bureau of Animal Industry of the United States Department of Agriculture, has found that of four colts which were deprived of colostrum, three died within 48 hours, and the remaining one, in less than three weeks. All four were infected with *Shigella equirulus.* Of six colts fed the same basal ration plus horse serum, all survived and grew normally throughout the experimental period of six months. The authors wish to express their indebtedness to Dr. Earle for permission to use this information, and for other help in revising the section on colostrum.

A review of the work in this field up to 1929 has been published by Braun et al.[27] Much evidence is given in this review which indicates that, although colostrum is less important as a means of furnishing immunity against infection in the case of human beings than in that of cattle, it may, nevertheless, sometimes provide the human infant with antibodies which act as a supplement to the forms of immunity acquired through the placenta ([27] p. 1132).

The various investigations of the immunological properties of colostrum have been unusually free from controversy and contradiction. Williams and Traum, however, have published statements indicating that they doubt whether colostrum has any immunological importance in the case of cattle, and Porcher has put forward a theory which would imply that the secretion of colostrum is a more or less accidental occurrence.

The following statement from Williams [280] indicates the character of his opposition to the views of other investigators. Williams says—"Notwithstanding the assertions of numerous teachers regarding feeding, the necessity for feeding colostrum to a calf is a myth. Experimentally, I have fed many calves upon boiled milk from birth, and colostrum can not be boiled without coagulating. While a calf can be well grown experimentally upon boiled milk from the outset, it requires very close watching and skillful handling in most instances. I prefer that for the first eight or ten days the calf should be fed the very small ration (2 per cent of body weight) of raw milk from its dam. During this period there is

confessedly the danger from infections borne in her milk, but under usual conditions this is more than counterbalanced by its content of protective substances of a highly essential character."

Traum,[263] influenced somewhat by Williams, sought to show that there was no difficulty in raising thriving calves without feeding colostrum, his basic problem being to free a herd from tuberculosis. In 1923 he again maintained [264] that as many healthy cows could be matured without colostrum as when this was fed, but stated that his records were incomplete. Elsewhere, in this review, he adhered firmly to a conviction of the necessity for feeding colostrum in order to insure adequate antibody transmission from dam to offspring.

Porcher's contention [218] that colostrum is a product of retention rather than an actual secretion, being the result of a phagocytosis of previously produced milk, should also be mentioned. By the injection of sterile milk into the peritoneum of guinea pigs he claims to have induced the formation there of a substance bearing a strong histological resemblance to colostrum. But there seems to have been scarcely sufficient experimental evidence adduced to support the assumption that these results really explain the formation of colostrum in the mammary gland of a cow.

General Biological Experiments on the Nutritive Properties of Milk

Experiments on the nutritive properties of milk, like other nutritional experiments, are of two types. One of these types is planned primarily from the chemical point of view wherein the investigator has in mind either the relation between the chemistry of milk and its nutritive properties, or the nutritive properties of some particular chemical constituent of milk. The other type is planned from the biological point of view wherein the investigator takes milk and other natural foods as he finds them, and studies the effects of milk alone or of milk combined with other products on the general health of his experimental animals. The advantages of the first type of experiments are obvious, but the difficulties connected with them are perhaps not quite so generally realized. The purification of foods and the determination of the metabolic activities of animals require so much labor and expense that it is seldom possible to carry out experiments of this type on large numbers of animals, or to continue them for very long. The history of the science of nutrition has shown that it often takes a long time for the full effects of given rations to show themselves in animals, and that different individual animals frequently react very differently to the same rations. It has been necessary, therefore, again and again to test out and revise, through the biological type of experiment, the conclusions drawn from the chemical type; and the state of knowledge at any given time is very imperfectly realized unless the results of both types are studied. In the following account the experiments of the general biological type will be considered first, and

afterward those in which the relationship between the nutritive properties of milk and its chemical composition have been kept more strictly in view.

Milk as a food for sucklings. Lane-Claypon [163] has given a good review of work which bears on the results obtained when young mammals are nourished on the milk of foreign species. The general conclusions from this work are that young mammals do not thrive as well on the milk of foreign species as on that of their own mothers, and that the deleterious effects of the foreign milk are more marked the earlier it is substituted for the mother's milk.

Very interesting results bearing on this last point have been reported by Moro [197] who found that of guinea pigs taken away from their mothers immediately after birth, 80 per cent died; of those left one day, 30 per cent died; of those left three days, only 10 per cent died; and of those left for longer periods, all lived. In the light of what is known regarding the secretion of colostrum at the beginning of lactation and of its effect in protecting the new-born mammal against bacterial invasion, it seems highly probable that the great mortality among guinea pigs which are taken away from their mothers immediately after birth is due to their being deprived of colostrum. Indeed, the recent development of knowledge regarding the function of colostrum suggests that much of the difficulty in nourishing young mammals on the milk of foreign species may be connected with their failure to get the colostrum secreted by their mothers, and it is highly desirable that the subject should be reinvestigated with this point in mind.

The work reviewed by Lane-Claypon,[163] however, gives good reason to believe that the milk of certain species is not suitable for the nourishment of the young of certain others, quite apart from the colostrum question. It is very difficult, for instance, to bring up young rabbits or kittens on cow's milk, whereas human infants and puppies do fairly well on it. Lane-Claypon has carried out an extensive investigation on the results obtained in raising babies on cow's milk and finds that, although babies so fed do not at first gain weight quite so fast as those nursed by their mothers, they nevertheless later regain all the ground lost at the beginning. Kuttner and Ratner [160] have carried out an investigation which indicates that human infants frequently survive even when they are entirely deprived of colostrum, and it would seem, therefore, as if human infants were able to withstand the substitution of a foreign milk for that of their mothers even from the very beginning of their lives better than most other mammals.

Attempts to nourish young mammals with the least possible amount of milk of any sort have been carried out chiefly with cattle, the object being to discover ways of reducing the expense of raising calves. Experiments of this kind have been carried out by Fraser and Brand,[94] Lindsey,[170] and others. Earlier experiments are referred to in the two bulletins just cited.

In all of these experiments, the calves are left with their dams for a day or so, so that they get colostrum. They are then fed for a few days on whole milk and afterward for a longer period on skim milk. The ex-

periments have consisted largely in studying and recording the effects produced by substituting other foods for the milk at various periods.

When it is not necessary to practice great economy, calves at present are often fed about 10 pounds of whole milk daily for the first two weeks of their lives and then changed gradually over to skim milk. From the age of about two weeks they are offered grain and hay, and are generally eating a good deal of these materials by the time they are six months old. Under such treatment they ought to make average daily gains in body weight of between one and two pounds.

Fraser and Brand [94] studied the effects of reducing both the whole milk and the skim milk. Three calves received only 17, 18, and 21 pounds, respectively, of whole milk altogether after the first four days of their lives. The authors found, however, that "changing so early to skim milk had a tendency to derange their digestions at first, and they did not do well afterward even though the skim milk was continued longer than would have been necessary had they been given a better start on the whole milk." Three other calves, after having been for about two weeks on whole milk, received altogether only 117, 166, and 176 pounds of skim milk each, and were then changed to a ration of clover hay, oats, corn, and wheat bran with either linseed oil meal or ground flax seed. They "developed a feverish condition in their digestive tracts," and "were decidedly thin at two months of age," but, with careful feeding they later developed satisfactorily and survived. The other three calves, which got more skim milk with very small amounts of whole milk, also finally developed into satisfactory cows. Fraser and Brand consider that calves can be raised satisfactorily with 140 to 200 pounds of whole milk and about 500 pounds of skim milk altogether, although the animals under these conditions gained less than a pound of body weight daily for the first six months.

Lindsey's experiments [170] were not very different from those of Fraser and Brand. He worked with three commercial calf meals, with six which he devised himself, and with one devised by Hayward of the Pennsylvania station. Hayward's meal contained between 20 and 25 per cent of skim milk powder. The calves which got this last meal received no skim milk in addition; the others got between 5 and 10 pounds daily for about the first six months.

In recent experiments carried out in the Bureau of Dairy Industry, and still unpublished, calves were allowed to take the colostrum secreted by their dams for the first 2 to 4 days of their lives; were then separated from their dams and put on a ration of skim milk supplemented either with 20 cc. of cod liver oil or 16 milligrams of carotene daily. The feeding of grain and timothy hay of poor quality was started when they were about three weeks old. On such rations the calves have remained in good health and grown satisfactorily. These results indicate that the chief reason for feeding whole milk in the first month of a calf's life is to supply sufficient vitamin A, which the calf cannot get at that period, as it can later, by eating hay. If sufficient vitamin A is supplied in the

form of carotene or cod liver oil, the feeding of whole milk can be dispensed with.

The experiments on reducing the quantity of milk fed to calves, which have been carried out up to the present time, indicate that calves require some easily assimilable form of vitamin A for the first month of their lives, and that, unless they receive about 2,000 pounds of skim milk in the first 3 or 4 months, they are likely to make small gains in weight and to have more sickness than calves fed according to the best recognized methods. There is still no complete and satisfactory explanation as to why the young mammal is so highly dependent on milk long after the end of the colostral period.

Milk as a food for mammals which have passed the suckling stage. The experiments in which milk has been fed to mammals which have passed the suckling stage, either as the sole diet or as a part of the diet, are legion. They group themselves rather naturally as will be apparent as the discussion proceeds. The experiments which will be described, unless otherwise noted, assume the use of cow's milk of a normal character, containing average amounts of the usual constituents, including vitamins A, B, D, and E, but not necessarily vitamin C. The latter, present at best in small amounts and likely to be wholly absent when the milk has undergone any heat treatment, is generally supplied from other sources. Furthermore, its need by rats and cattle, with which the experiments detailed here largely deal, has never been demonstrated.

Davenport,[50] in testing the effect of depriving ruminants of coarse feed, fed calves on a diet of skim milk, either alone or supplemented with grain. The animals exhibited an enormous appetite, "followed by enlargement and stiffening of the joints, spells of dizziness and difficult locomotion, all followed by periods of relief and finally by a settled feeling of indifference to food." They grew well for three or four months, then more slowly, and at length their weights became nearly stationary, but underwent a sudden unexplained rise just before their collapse. Some of them made rapid and apparently complete recoveries upon the addition of hay or straw to the diet, even after seven months.

McCandlish[178] fed two calves whole milk until their death at the ages of 208 and 176 days. They grew fairly well for 2 or 3 months, then more slowly, and finally declined in weight. They showed an extraordinary appetite for salt and a tendency to have fits similar to those of an epileptic nature. At post mortem the bones of one were found to be very flexible, while some of those of the other appeared to have been broken and then healed.

Huffman and Robinson[185] fed calves on milk and on milk supplemented with various materials. Of 14 animals 11 succumbed, 10 of these before the 300th day of the experiment. Most of them exhibited the typical symptoms observed in calves fed milk for a prolonged period of time, namely, nervous disorders, convulsions, stiffness of limbs, etc., and a lowered blood calcium. The 3 remaining calves were the only ones which had received at some time during the experiment an iron supple-

ment (syrup of iron phosphate) in their diets. They showed no marked symptoms, had normal blood pictures, and survived until removed from the experiment on the 501st, 611th, and 710th days.

Daniels and Stuessy [45] were unsuccessful in attempts to raise rats on raw cow's milk. Growth was slow and below normal and the animals could not be kept alive on this diet. Milk heated to boiling proved to be inadequate for normal growth and resulted in reproductive failure.

Winfield [281] fed dried milk to rats from before the normal time of weaning for as long as 16 months in some cases. A normal rate of growth occurred until one-half to two-thirds of the adult weight was reached when it became slower than normal or the weight remained stationary. It is stated that the animals remained healthy throughout the experiment, but that reproduction was abnormal and that none of the young borne survived.

Mattill and Conklin [185] found that in rats fed from weaning on fresh cow's milk initial growth was good, but this was retarded between the 50th and 100th day of life. Reproduction did not take place. Dried milk afforded much better growth but here also reproduction failed to occur.

Gibson and Concepcion [96] found that the exclusive feeding of fresh or autoclaved cow's milk to pigs and dogs induced certain symptoms of beri-beri,—degeneration of the peripheral nerves, persistent oedema, etc. Little difference was observed between the results of feeding fresh and autoclaved milk.

Moro [197] fed guinea pigs on raw and sterilized cow's milk. The animals showed characteristic signs of an "alimentary intoxication"; death occurred in from 3 to 5 days. Rabbits fed on cow's milk died after several weeks. Death took place on both the raw and sterilized milk diets.

Evidently milk is not a complete food beyond the early stages of life, and using it as the sole food for any great length of time after the suckling period leads to various pathological conditions. In the experiments so far described, only those symptoms have been reported which are obvious to the general observer, but in certain other experiments it has been observed that one of the prominent results of exclusive milk feeding beyond the suckling stage is anemia. As a result of this observation a great deal of work has been done on the relation between milk feeding and anemia, from which it has been made clear that milk is deficient in iron and copper, both of which appear to be necessary for the building of blood. A good review of the subject of iron and copper in food and nutrition, with numerous references to the literature, has recently been given by Sherman.[246] In this review is included a present-day classification of the various types of anemia. For good reviews of the recent literature dealing with the anemia produced by milk feeding the reader is referred to those of Smith [5a, 6b] on nutrition.

Relation between the feeding of milk and anemia. The term anemia is ordinarily taken to mean a deficiency in the hemoglobin content or in the red cell count, or in both, of the blood as a whole.

Anemia and iron have long been associated in the majority of the

investigations dealing with this condition, for it has been known for many years that iron is an essential constituent of hemoglobin, the pigment of the red blood cells. Iron is also a constituent of the chromatin substances of the body cells, and small amounts of this element, it is believed, may take part in some of the oxidation-reduction processes occurring in the organism. According to Sherman [246] the adult human body contains about 0.004 per cent of iron, and cow's milk about 0.00024 per cent.

Bunge,[28] in his studies on the assimilation of iron by the suckling, and Abderhalden,[1, 2] in investigations on the relation of the ash of the new-born to that of the ash of the milk of the same species, found that, with the exception of iron, the relative proportions of the mineral elements were very nearly the same in the two. The ash of the new-born contained several times the amount of iron in the milk ash. This was found to be true of those animals which depended on milk alone for their nourishment during the suckling period. In the case of the guinea pig which begins to feed upon green foods almost immediately after birth, the difference in iron content was not nearly so large.

Bunge [29, 30] showed also that the iron per kilogram of body weight was highest at birth and decreased throughout the suckling period to a minimum toward the end of this period; the absolute quantity of iron remained fairly constant during this time. Guinea pigs contain only a small store of iron at birth.

Abderhalden [4] found that the absolute quantity of hemoglobin increases throughout the suckling period; the hemoglobin per kilogram of body weight decreases throughout this time to a minimum toward the end of the period. This work was carried out upon rats and rabbits. Moreover, using the figure for the iron content of horse hemoglobin and Bunge's figures for the iron contents of rabbits at ages closely corresponding to the ages at which he determined the hemoglobin contents of his own rabbits, Abderhalden was able to calculate the content of iron present not-as-hemoglobin, and while noting that such values were necessarily not accurate, showed that the content of iron present not-as-hemoglobin possessed a maximum value immediately after birth and decreased during the suckling period as the absolute quantity of hemoglobin increased.

As soon as the iron-poor milk diet is changed to an iron-rich one (green plant material), both the absolute quantity of hemoglobin and the quantity per kilogram of body weight rise rapidly.

Thus those animals which depend for some time after birth on milk as the sole source of nourishment, contain at birth a reserve store of iron which they use together with that of the milk for the formation of hemoglobin during the suckling period. Since at the end of the suckling period, the body iron is very largely in the form of hemoglobin and the reserve supply of iron at most very small, a continued negative iron balance caused by a low iron intake must result eventually in a lack of hemoglobin and hence in some degree of anemia.

That anemia develops in animals which are kept on exclusive milk diets for some time beyond weaning was shown in 1900 by Abderhalden.[3]

His observations in this respect have been confirmed in recent years by numerous investigators. In a long series of experiments with large numbers of animals of several different species, Abderhalden showed that the young kept for about three weeks to a month from the time of weaning (guinea pigs shortly after birth) on a diet of cow's milk or of cow's milk and rice, possessed a much lower absolute and percentage amount of hemoglobin in the body than did animals on natural-food diets of vegetables, meat, etc. The addition of ferric chloride or hematin to the iron-poor foods led to distinct improvement but did not render these diets nutritionally equivalent to those of natural foods as regards their hemoglobin-producing properties.

Scott [228] found that young rats bred from mothers on a diet of whole milk and white bread were anemic; when maintained on this diet the anemia tended to disappear in time but recovery was more rapid when green plant material or ferrous chloride was added to the diet. Rats on a diet of white bread, ferrous chloride, skim milk, and palm kernel oil showed an anemic condition compared to rats on a diet of white bread, ferrous chloride, and whole milk. It was concluded that some factor associated with butterfat influenced hemoglobin formation.

The experiments which have just been described show that keeping animals on an exclusive milk diet, after the suckling period is over, results in anemia; that the anemia so caused may be relieved to some extent by adding inorganic salts of iron to the milk, but that it is not possible in this way to cure or prevent the anemia altogether. Until very recently the evidence stood in favor of the view that milk was deficient in both iron itself and in certain organic compounds necessary for its utilization in the formation of hemoglobin.

Support was apparently given to this view by the first results of the work of Hart, Steenbock, Elvehjem, and Waddell [110] at the University of Wisconsin, who in 1925 reopened the question of the anemia resulting from exclusive milk feeding beyond the suckling period. These workers studied the anemia so produced in rabbits and found that this condition could not be permanently relieved by adding inorganic iron (Fe_2O_3) to the milk. Additional results were obtained which led the investigators to believe that animals could not build hemoglobin from milk unless there were added to it both iron and an organic compound related to chlorophyll.

An entirely new light was thrown on the question by the further work of Hart and his collaborators [111] who found that the anemia produced in rabbits by a diet of whole milk plus small amounts of ferric oxide could be completely relieved by the addition to the diet of the ash of either lettuce or cabbage. At the time their work was written up for publication they had succeeded in keeping young rabbits entirely free from anemia and growing at the normal rate for periods of eight or nine months on diets consisting exclusively of milk plus small additions of ferric oxide and the plant ash. On diets of milk plus ferric oxide alone, rabbits of the same litter as those previously mentioned had become anemic and died in the course of three or four months. Also it was found that the practi-

cally iron-free ash of an alcoholic extract of cabbage was potent in preventing the development of the anemia produced on the whole milk–ferric oxide diet. An impure soluble $FeSO_4.7H_2O$ proved to be effective in curing the anemia, whereas purified preparations of $FeSO_4.7H_2O$ were much less effective but more so than Fe_2O_3.

It therefore became apparent that the deficiency in milk which led to the nutritional anemia was inorganic rather than organic in character.

Similar and more extended results were obtained later on rats by Waddell, Elvehjem, Steenbock, and Hart.[270] It was found that whereas each of several purified iron salts when fed in small amounts (0.5 mg. iron per day) did not materially increase the level of hemoglobin in rats made anemic by exclusive milk feeding, the same amount of iron fed as the ash or as the hydrochloric acid extract of the ash of dried liver, or of dried lettuce, or of yellow corn was very effective in curing the anemia.

As a result of their experiments on rabbits and rats the Wisconsin workers were led to postulate the existence of some inorganic substance or substances which, in addition to iron, are vitally concerned in the formation of hemoglobin and which along with iron bring about correction of the nutritional anemia produced by exclusive milk diets.

In the next paper of their series, immediately following the above work on rats, Hart, Steenbock, Waddell, and Elvehjem[112] reported the discovery that copper when fed along with iron brought about striking remission of the anemia. Both a liver preparation and its ash were effective in supplementing iron in curing the anemia produced in rats by feeding milk alone. When the hydrochloric acid extract of the ash was treated with H_2S, followed by NH_4OH-$(NH_4)_2S$, the anti-anemic potency was concentrated in the H_2S fraction. Copper, as one of the elements in the H_2S fraction, was highly effective when 0.05 or 0.1 mg. of it (as $CuSO_4.5H_2O$) and 0.5 mg. of iron (as $FeCl_3$) were fed six times per week as supplements to the milk. The ferric chloride used was a purified preparation which had been found previously to be ineffective when fed in the same amount and at the same rate.

Almost simultaneously McHargue, Healy, and Hill[179] reported a short experiment with rats, from the results of which the authors inferred that copper has an important function in the formation of hemoglobin and in the metabolism of red-blooded animals.

This discovery has led to renewed interest and much experimental work in the field of nutritional anemia. The strikingly beneficial effect of feeding iron and copper together in the prevention and cure of the anemia produced in rats by exclusive milk feeding has been amply confirmed by the further work of the Wisconsin investigators[272, 273] and by the results obtained by numerous other workers, e.g., Titus, Cave, and Hughes,[260] Beard and Myers,[13] Underhill, Orten, and Lewis,[266, 203] Krauss,[155, 156] Lewis, Weichselbaum, and McGhee,[166] Keil and Nelson,[146] and Mitchell and Miller.[192]

It has been reported by some workers, e.g., [14, 15] that iron alone, in sufficient amounts, is effective in bringing about either the cure or pre-

vention of the milk anemia of the rat; and that small amounts not only of copper but also of each of a whole series of other metals,[198] and certain amino acids (or their sodium salts or hydrochlorides [58, 59, 89]) are effective supplements to iron in relieving the condition. Other investigators have been unable to confirm these findings.[271, 273, 146, 78, 147, 266]

The evidence shows conclusively that copper is a necessary factor in the production of hemoglobin. Among all of the substances which have been investigated with regard to their ability to supplement iron in promoting the cure and prevention of the anemia of the rat due to feeding an exclusive milk diet beyond the time of weaning, copper is the only one which has been effective in the hands of the great majority of the workers in this field.

When copper alone was fed to rats on a whole milk diet the anemia was not prevented or cured.[261, 274, 226, 256]

The exact mechanism of the effect of copper in supplementing a milk-iron diet to bring about blood formation is not known.

Copper has been found to occur in the blood of a number of species of red-blooded animals,[178, 275] but it does not seem to be a constituent of the hemoglobin molecule.[48, 71] Various views have been taken by different workers as to the rôle of copper in blood formation with reference to its effect upon the reticulocytes, erythrocytes, and hemoglobin.[16, 226, 256, 227, 76] The absorption and storage of iron can take place independently of copper but copper does function in the utilization of iron for the production of the blood hemoglobin. When a pure inorganic iron salt was added to the whole-milk diet of anemic rats which had been well depleted of their bodily store of iron, no rise in the hemoglobin content of the blood resulted but there occurred a decided increase in the iron content of the liver and spleen which was more or less proportional to the amount of iron fed. Subsequent replacement of the iron added to the milk by copper led to a temporary increase in the hemoglobin content of the blood, which was accompanied by a depletion of the iron store in the liver.[75, 38, 44, 148] Similarly, the feeding of copper alone evidently results in a bodily store of this element which can be utilized in the production of hemoglobin when iron is fed subsequently. When rats were placed at weaning on a diet of whole milk plus a pure iron salt ($FeCl_3$), anemia with a low hemoglobin value developed; other rats fed similarly on a whole milk–copper sulphate diet also developed anemia, but when the copper sulphate in the diet of the anemic rats was replaced by a supplement of the pure iron salt, the hemoglobin increased immediately and returned gradually to practically the normal value.[261]

Elvehjem and his associates have studied the availability of iron from different sources for hemoglobin regeneration. Rats were rendered anemic by feeding an exclusive milk diet. Copper sulphate was supplied along with the iron supplements in the recovery period. It was found [74] that ferric chloride was much superior to hematin as a source of iron for hemoglobin regeneration in the anemic rats. In addition, the iron content of the livers from the different animals indicated that the iron from ferric

chloride was more readily absorbed than that from hematin. Later, Elvehjem, Hart, and Sherman [78] determined the degree of availability of iron in different types of iron salts and in a few food materials (a) by a chemical method (Hill's dipyridyl method) and (b) by animal feeding. They found that "practically all the iron in ferric chloride, ferric pyrophosphate, ferric glutamate, and ferric hypophosphite reacted with dipyridyl. These salts also supplied readily available iron for hemoglobin formation. The iron in glutamic acid parahematin failed to react with dipyridyl and showed a very slight availability by animal feeding. 47 per cent of the total iron in samples of wheat and yeast and 57 per cent of the total in a sample of oats was found to react with dipyridyl." From the feeding experiments they estimate an availability of iron approximately equal to those values for oats and yeast, and suggest that the dipyridyl method may be a valuable means of determining available iron in foodstuffs.

The hemoglobin producing properties of various food materials fed as supplements to the whole milk diets of anemic rats have been investigated. References to this work are cited in the articles of Rose, Vahlteich, and MacLeod,[221] and of Elvehjem, Hart, and Sherman.[79]

As previously mentioned in this discussion, Bunge showed that the young mammal is provided with a reserve store of iron at birth. Likewise, a bodily reserve of copper seems to be indicated. McHargue [178] found the concentration of copper in the total dry matter of rats 12 hours old to be about twice as great as in that of the carcasses (minus the intestinal tracts) of rats having a live weight of about 100 grams; the percentage of copper in the liver of a new-born calf was approximately 15 times as great as in that of a mature ox. The results of Lindow, Peterson, and Steenbock [169] showed that the percentage of copper in rats on a stock diet was highest at birth and decreased continuously up to about 85 days, while the absolute amount increased with age. As with the calf, a concentration of copper in the liver of the new-born several times as great as that in the liver of the adult of the same species, has been observed with other mammals,[44] including the human.[151]

Ramage, Sheldon, and Sheldon [216] have recently reported the results of a spectrographic investigation of the metallic content of the human liver during intra-uterine life and childhood. The livers were from children whose deaths were caused by a great variety of diseases. The results show that in this organ, the percentage of iron increases during intra-uterine life, falls rapidly during the nursing period, and then increases when a mixed diet is taken. The percentage of copper follows approximately the same course, but does not increase subsequent to the nursing period.

Elvehjem has recently published an article in which he considers the question whether human beings would often be benefited by the addition of iron and copper salts to diets which have been considered adequate in the past.[76] The hemoglobin content of the blood of approximately 1,000 children brought to the child health centers in Madison, Wisconsin, has

been studied. Severe anemia was encountered in very few cases. It was only when all modern methods of infant feeding were completely disregarded, and a diet of milk alone continued for 5 or 6 months that exceedingly low hemoglobin readings were obtained. However, it was found that the hemoglobin content of the blood of supposedly normal children varied, and could often be increased by the addition of iron and copper salts to the diet. Some evidence is cited which indicates a correlation between the prevalence of children's diseases and low hemoglobin content of the blood.

The question whether it would be advantageous to add small amounts of iron and copper salts to the diet of infants as a matter of routine is a very interesting one, but should be much further studied before definite recommendations are given. As will be brought out later in this chapter, other metabolic processes, such as the deposition of calcium and phosphorous salts in the bones, can be made to take place at a more rapid rate than on ordinary normal diets by the addition of newly discovered dietary factors to the food. But the fact that it has recently been discovered how to accelerate some particular metabolic process such as hemoglobin building or bone building is not sufficient warrant for concluding that it would be generally advantageous to force that particular process to go on at the maximum rate. It has often turned out that an element or compound which is nutritionally advantageous from one point of view is deleterious from another; for instance, ferrous sulphate, which can be used for hemoglobin building, may have a deleterious effect on vitamin A.[177] It seems wise, therefore, not to make general recommendations about the addition of particular nutritional factors to what have long been recognized as fairly satisfactory diets until the effects of such particular factors have been very thoroughly tried.

Variation of iron and copper in milk with the food of the lactating animal and with the administration of iron and copper with the food. The iron content of cow's milk is low. Sherman [246] states that the results of iron analyses of samples of cow's milk of widely different origin varied from 0.0002 to 0.0003 per cent and averaged 0.00024 per cent.

This same author,[282] in a review of iron metabolism published in 1907, cites a number of experiments (principally upon goats) which indicate that the iron content of the milk can be increased by the administration of iron in forms other than those occurring in ordinary foods. It was not established whether the iron thus introduced was of nutritive value similar to that ordinarily present in the milk.

In some recent carefully planned experiments designed to answer this question, Elvehjem, Herrin and Hart [70] found that the administration of iron oxide (Fe_2O_3) or ferrous sulphate ($FeSO_4.7H_2O$) in quantities which increased the iron intake of goats (on a basal ration of grain and alfalfa hay) five-fold, did not increase the iron content of the milk; adding fresh green cabbage along with Fe_2O_3 had no effect. Feeding Fe_2O_3 or Fe_2O_3 plus green cabbage as an addition to the diet did not render the

milk capable of preventing anemia in young rabbits placed upon it as the sole article of diet. The iron content of the milk from individual cows varied as much as 100 per cent; the average iron content of the milk of three cows receiving alfalfa hay in the ration was not different from that of three cows receiving timothy hay in the ration. In a previous article [69] these workers point out that the older methods of iron determination are not satisfactory when applied to such material as milk.

Similar results were obtained [72] with regard to copper. When the copper intake of cows fed a normal ration of alfalfa hay, silage, and a grain mixture was increased five-fold through the addition of copper sulfate ($CuSO_4.5H_2O$) to the ration, no increase was observed in the copper content of the milk; the same result was obtained with goats on increasing the copper intake five- or ten-fold by adding copper sulfate to their diet.

The Wisconsin investigators give 0.000015 per cent as the copper content of cow's milk. This value is low compared to the corresponding values of the great majority of about 160 common food materials analyzed by these workers.[168]

Copper analyses were carried out by these investigators [72] on samples of milk collected from herds located in 13 states, representing practically every region of the United States. It is stated that the herds were receiving rations typical of their particular section of the country, and that, in most cases the roughage, at least, was home grown. Very little difference was found in the copper content of these samples, the values, in milligrams per liter, ranging from 0.123 for the milk from North Carolina to 0.184 for that from Texas, and averaging for all 13 states, 0.147.

It is, however, well known that milk may take up additional iron and copper by contact with these metals.

Long-continued experiments on the biological effects of diets containing milk. Sherman and his collaborators have carried out a very interesting series of experiments which bear on the nutritive properties of milk, using white rats as their experimental animals. They first tried the effects of feeding these animals on white bread alone and on white bread supplemented with various other single natural food substances.[283] The bread was made from patent flour with water and the minimum amounts of yeast, sugar, salt and lard. It contained therefore liberal amounts of carbohydrate and fairly good protein, but was low in mineral elements and vitamins. Young rats fed on this bread alone failed to grow, and died in the course of about six weeks. The bread was then supplemented with meat, apple, turnip, or milk, each of the above mentioned foods being added in the proportion of one calorie of the added food to four calories of the bread. On bread and meat the rats made some growth, but died in about seven weeks; on bread and apple they did not grow and lived for about 10 weeks; on bread and turnip they grew slowly from a weight of about 25 grams to a weight of about one hundred grams in the course of six months; while, on bread and milk they made a

much more rapid growth and survived throughout the experimental period (170 days).

On further study, however, it was found that the bread and milk diet above described was not entirely satisfactory. Female rats, which were fed on it from the time of weaning up to sexual maturity, gave birth to young but failed to raise any of the young beyond the suckling period. Substituting ground whole wheat for the white bread made it possible for the rats to suckle their young successfully.

After having reached this point in their investigations Sherman and his collaborators proceeded to work toward devising a simple ration on which a rat colony could be kept indefinitely with optimum or nearly optimum results in regard to growth, reproduction, and general health. They found that milk powder produced as good results as liquid milk, and that such heating as occurred to milk when it was subjected to baking temperature as one of the constituents of bread did not injure its nutritive properties for rats. They then tried using different proportions of whole milk powder and ground whole wheat in their ration, and found that a ration composed of one-third by weight whole milk powder and two-thirds whole ground wheat produced better results than one composed of one-sixth whole milk powder and five-sixths whole ground wheat. (In this latter ration the wheat supplies about four-fifths of the calories, and the milk about one-fifth.) In addition to these proportions, they tried the effects on growth of rations containing one-half and two-thirds whole milk powder.[284] While growth on the rations containing these larger proportions of milk was slightly more rapid than on that containing one-third whole milk powder, the results with the last-named ration were so nearly optimal that it was selected to be used extensively in later experiments.

A careful comparison has been made between the results obtained with the ration made up of one-sixth whole milk powder and five-sixths whole wheat with sodium chloride equal to 2 per cent of the wheat, and those obtained with one-third whole milk powder and two-thirds whole wheat with sodium chloride equal to 2 per cent of the wheat. The investigators call the first of these Diet A, and the second Diet B, and these designations will be used in the subsequent discussion.

Rats can be maintained for many generations on either of these diets. Sherman and Quinn [241] reported that some of their rats have reached the fourteenth generation on Diet A and the seventeenth generation on Diet B. On Diet B, however, the rats grow faster, begin to reproduce earlier, continue reproducing to a later period of life, give birth to more young, and are successful in suckling a larger proportion of the young born. The following quotation from Sherman gives a good idea of the difference in the results produced by the two rations. "As explained above, the initial animals of Series II were all from one litter, divided into two equal groups of one male and three females each. Starting thus exactly simultaneously and with precisely the same family history, each group and all its descendants were kept on its respective diet and allowed

to multiply without restriction at such rates as the nutritive conditions resulting from their respective diets would permit, until 1 year from the date of birth of the original animals. The result was that at this time those on Diet A had a total of 77 descendants, while those on Diet B had 361 descendants, an increase of 368 per cent in the cumulative breeding record on Diet B over that on Diet A." [237]

In still another investigation, Sherman and his collaborators [238] have studied the importance of butterfat in Diet B and in various modifications of it, some of which had a high and some a low butterfat content. The diets high in butterfat consisted either of Diet B itself or of a similar diet in which the whole milk powder was replaced by an equivalent mixture of skim milk powder and butter. The diets low in butterfat contained two-thirds ground whole wheat and one-third skim milk powder or a mixture of skim milk powder with lard or coconut oil equivalent in fat content to whole milk powder. In all cases sodium chloride equal to 2 per cent of the weight of the wheat was added.

The results on the two high butterfat rations were similar, as were also those on the three low butterfat rations. They may be summed up in the following statement largely quoted from Sherman. A total of 17 females and 5 males were continued until death on each of the two types of diets. Each animal on the diet high in butterfat was matched against an animal of the same sex and litter on the diet low in butterfat, the parallel groups having exactly the same inheritance and previous nutritional history, and being kept under conditions alike in all respects except that one received a diet richer in butterfat than the other. The average maximum weight of the 17 females on the high butterfat diets was 259 grams, and, for the 5 males, 352 grams; while for the females on the low butterfat diets the average maximum weight was 181 grams, and, for the males, 244 grams. The average length of life on the low butterfat diets was 369 days; on the high butterfat diets, 746 days. The 17 females on the high butterfat diets had a total of 477 young, of which 264 were successfully suckled and weaned at a fully average size and vigor. The 17 females on the low butterfat diets had 31 young, none of which survived more than 2 days after birth. These 17 females did not develop xerophthalmia, but tended to die of lung infections in early adult life.

These results of Sherman's, taken all together, point to several important conclusions. One of these is that the feeding of deficient rations to colonies of animals may lead to a high incidence of disease. Milk contains factors which render animals less susceptible to infection. Further, reproduction is particularly likely to be unfavorably affected by dietary deficiencies. But a diet may be adequate for the support of a species, may permit it to reproduce through an unlimited number of generations, and may, nevertheless, restrict the rate of reproduction to far below the optimum.

The work with the rats on Diets A and B is being continued and some further aspects of it are given in two recent articles (Sherman and Campbell, 1930,[244] Sherman, 1933 [247]). In August 1933 the colony on Diet A

had reached the thirty-fourth generation. The longevity of the rats on the two diets has been studied and it has been found that on Diet B the infant mortality is reduced and the average length of life of the adults is increased about 10 per cent. More rats live to each of such ages as 800, 900, and 1000 days on Diet B than on Diet A.

The greater longevity on Diet B is associated with a more rapid rate of growth, and the conclusion drawn from certain experiments that there is an inverse relation between rapid growth and longevity cannot, therefore, be regarded as generally correct.

Summary of experimental results on the general nutritive properties of milk. Our present general knowledge regarding the nutritive properties of milk may be summarized as follows: During the first few days of the life of the young mammal the colostrum secreted by its mother in that period plays a quite unique part in maintaining its life and health. Contrary to all the ordinary physiological rules, unchanged protein passes from the blood of the mother through the gland cells into the mammary secretion, and thence through the cells of the intestinal tract of the offspring into its blood, where it affords a protection against bacterial invasion. For the first few days of life, therefore, the young mammal, in many species at least, is peculiarly dependent on the mammary secretion of its own mother, and not even the milk of another female of the same species can take the place of this secretion.

As the young mammal gets older, it rapidly becomes less dependent on the mammary secretion of its mother, and finally reaches a stage where it can be nourished without milk at all. There is an intermediate period, however, varying in length in different species, during which it is generally more difficult to nourish the young mammal on the milk of other species than on that of its own, and very difficult if not impossible to keep it alive and in good health without milk at all. The reasons for this situation and the details of the situation itself are a fertile field for investigation in the light of our recently acquired knowledge of nutrition.

In many species the young mammal thrives for a period of several weeks or months on milk alone. But, if continued on milk alone for long after the natural suckling period, it develops anemia and other pathological conditions, ceases to grow at the optimum rate, and shows various kinds of abnormality in reproduction. The experimental evidence as it stands at present indicates that milk can be made a nearly complete food for the adolescent and adult members of one mammalian species at least by the addition of small quantities of salts of the elements, aluminum, copper, fluorine, iodine, iron, manganese, and silicon.[46, 148, 112]

A colony of rats has been kept in a thriving condition through thirty-four generations on a diet which contained only dried whole cow's milk, whole ground wheat, and sodium chloride; and there is every reason to believe that this diet would maintain these animals in good health through as many generations as might be desired.

Part Played by Milk in the Development of the Modern Knowledge of Nutrition

Insufficiency of highly purified foods. The beginning of the present century was marked by many experimental attempts to nourish animals on chemically purified food substances. All of these experiments were failures; the animals ate progressively less of the purified foods, lost weight, and died within short periods. The failures were often explained on the supposition that the monotony or tastelessness of the diet caused the animals to lose their appetite for it; but at the present time, a quite different explanation of these failures is accepted, largely as a result of the study of the dietary properties of milk.

Quite early in the course of these studies it was found that rats and mice could be kept alive and apparently in good health for periods of six months or more on diets of milk alone.[204] It was clear, therefore, that neither monotony of diet nor close confinement would cause a fatal loss of appetite in these animals, provided that the monotonous diet were properly selected.

Much further light was thrown on the science of nutrition and on the peculiar nutritive properties of milk by the experiments of Hopkins[127] published in 1912, and showing the extraordinary effects of a small amount of milk, added to certain rations, on the growth of rats. Figure 39 illustrates his results.

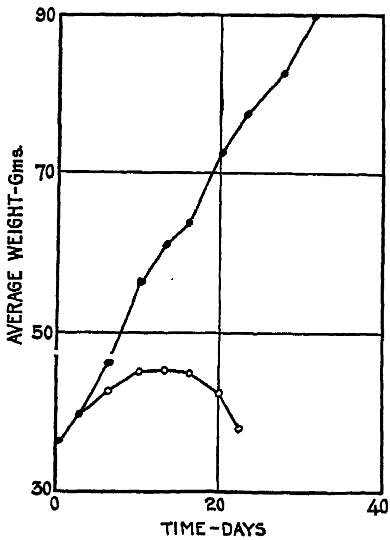


FIG. 39.—Lower curve, six rats on artificial diet alone. Upper curve, six similar animals receiving in addition 2 cc. of milk each per day. From Hopkins.[127]

The lower curve represents the average growth of six rats on a diet of casein 22, starch 42, cane sugar 21, lard 12.4, and salts 2.6. The salts were obtained by incinerating a ration which had been found to promote normal growth. By the twentieth day on this diet the animals were losing

in weight; a week later five of the six were dead. They ceased to grow at a time when their food intake was more than sufficient quantitatively to maintain normal growth. The upper curve represents the growth of six rats fed the same diet except that in addition each received 2 cc. of milk daily. This was fed separately so as not to affect the palatability of the rest of the ration. The milk solids constituted only 2.5 to 3.5 per cent of the total dietary. The total consumption of food per unit of weight and its digestion were nearly the same in both cases. The added milk could not have furnished enough of any of the known dietary essentials to bring about the difference in growth.

The same effects on growth were obtained by adding protein-free and ash-free extracts of milk solids to the artificial diet instead of whole milk. An alcoholic extract of milk solids, for instance, produced the same effects on growth.

These results of Hopkins pointed strongly to the view that the failures to nourish animals on diets of purified protein, fat, carbohydrate, and inorganic salts were not due to confinement of the animals or to monotony or lack of palatability of the diets, but to absence from such diets of certain unknown chemical compounds necessary for health and growth. Hopkins had carried out some of the experimental work reported in the article above referred to a number of years earlier, and had drawn the conclusions above indicated as early as 1906.[126]

The work of Hopkins indicates that milk is a particularly good source of the unknown material necessary for nutrition, and, since 1906 a great deal of work has been done by many investigators which bears on the nature of this nutritive material contained in milk and on its presence in other foods.

Very important early work on this subject was published by Osborne and Mendel.[204] The primary purpose of this work was to determine the nutritive value of different proteins, and the animals were usually fed on rations made up of a single purified protein with addition of carbohydrates, fat, inorganic salts, and agar-agar to serve as roughage. It was found that rats could be maintained on such rations for from 50 to 286 days ([204] p. 51), but that young rats made no growth on them. Like other investigators, Osborne and Mendel found that milk and milk products caused a resumption of growth in young animals when added to or substituted for their artificial rations. They laid particular emphasis on the results obtained with "protein-free milk," which was prepared from nearly fat-free centrifuged milk by precipitating the protein and evaporating the remaining solution to dryness. When this residue was added to one of the artificial rations which contained a biologically complete protein, growth was resumed and continued for several weeks.

The above described work of Hopkins and of Osborne and Mendel combined with other work on certain nutritional diseases has led to our modern knowledge of the vitamins and of their presence in milk. Since its publication much further work has been done on the vitamins of milk, and on its other nutritive constituents; and it has turned out that milk

contains most of the materials which are necessary for nutrition in easily assimilable and nutritionally advantageous form. In giving an account of this work, it will be convenient to begin with that on the vitamins, and then to take up the nutritional work on the proteins and minerals of milk.

Work subsequent to that of Hopkins and of Osborne and Mendel has shown that milk contains two classes of unidentified nutritive essentials, the one soluble in water, and the other in fat and fat solvents. A diet must contain both of these classes of essentials or vitamins in order to support health and growth in young animals.[175, 205, 189, 176]

A vitamin may be defined as an organic dietary essential, which is neither a protein, fat, or carbohydrate, and of which only a very small quantity is needed to produce its effect in nutrition. A few years ago there were five generally recognized vitamins, which were called A, B, C, D, and E. More work is being done in this field of research, however, than in almost any other. It is now clear that most of these letters must be regarded as standing for groups of compounds. The old system of nomenclature will be used to a considerable extent in the following discussion, but it must be understood that the subject is in a state in which it is difficult to speak always with complete precision.

All of the vitamins are found in milk, as far as is known at present, but they are found also in many other natural foods. There are a number of excellent recent reviews of this field with numerous references to the literature, among which may be mentioned the text book of Sherman and Smith[245] and a recent publication of the Medical Research Council of Great Britain.[187] The following discussion will give only a rather summary account of this subject in so far as it bears on the nutritional value of milk.

Vitamin A. Almost simultaneously McCollum and Davis[175] and Osborne and Mendel[205] found that butterfat or the ether extract of butterfat or egg yolk contains the substance essential for growth which has since become known as vitamin A. It also occurs in cod liver oil. It does not occur in lard, is not associated with the fatty acids or glycerides of the fats containing it, is not destroyed when these fats are saponified in the absence of oxygen, and is found in the "unsaponifiable" matter thus obtained. It is not cholesterol, which forms a large portion of this fraction in these fats. Takahashi and coworkers[258] believed that it was an unsaturated alcohol closely related to cholesterol. Drummond and coworkers,[60] while believing that vitamin A might be an unsaturated alcohol, held that Takahashi's preparation of it was impure and that their own work was not sufficient to determine the question.

A good account of the pathological effects which are produced in animals by foods deficient in vitamin A is given in the review of the British Medical Research Council.[187] These are failure to grow, susceptibility to infections, particularly of the eyes and of the respiratory tract, failure in reproduction, and degeneration of certain parts of the spinal cord. One of the most common results of vitamin A deficiency is an infection of the eyes beginning in the conjunctiva, which is known as

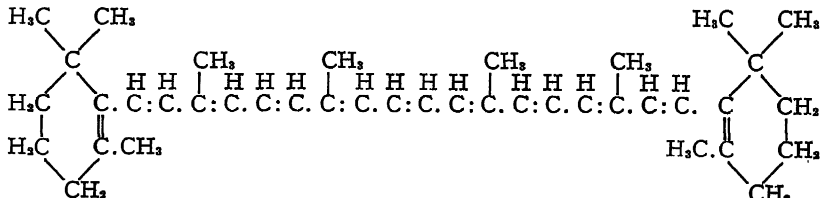
xerophthalmia. Other pathological conditions of the eyes are frequently observed in animals on A deficient diets; they are apparently not always connected with xerophthalmia. Attention should be called to the fact that the pathological effects of A deficiency have now been observed in many widely different species of animals. The following statement is not at all complete or exhaustive, but gives some idea of the situation. Xerophthalmia, as the result of A deficiency, has been observed in human beings, rats, rabbits, cattle, and chickens; night-blindness in rats and human beings; susceptibility to respiratory infections in rats, cattle, dogs, and human beings; failure in reproduction, in rats, cattle, and rabbits; and spinal cord degeneration, in dogs and swine. Failure to grow has been observed in all the species of animals that have been studied.

Davis and Moore have recently shown that over-doses of vitamin A may produce toxic effects.[51] To produce such effects, however, it is necessary to feed many hundred times the quantity of A that is required for growth and well-being.

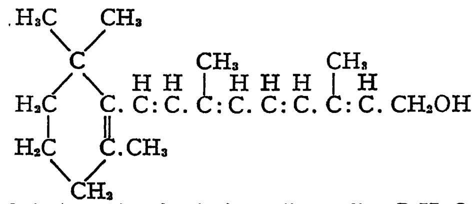
Vitamin A is determined quantitatively with young rats that have either ceased to grow or have developed xerophthalmia as a result of a deficiency of this factor in a diet otherwise complete. The Sherman unit of A activity, which has been made use of in many investigations, is that amount of A activity which will cause young rats to increase in weight at the rate of 3 grams per week in an experimental period of from 4 to 8 weeks under certain standard conditions. A fairly full account of how this determination is made is to be found in the text book of Sherman and Smith.[245b]

Vitamin A is stored to a considerable extent in the animal body, chiefly in the liver. There is very little in muscle meats; but kidney tissue may be 40 times as rich as muscle in its content of this vitamin, lung tissue more than 40 times, and liver tissue 200 to 400 times. The bodily store is greatly influenced by diet, and may greatly prolong the length of time that an animal may survive on a diet devoid of it.

Our knowledge of vitamin A has increased rapidly in the last four or five years. It is now generally recognized that carotene, one of the yellow pigments found in plant materials, is a potent source of vitamin A. This discovery has led to intensive work on the chemical nature, both of carotene and of the colorless form of vitamin A found in cod liver oil, and chemical formulas have been proposed for both substances, which are probably nearly correct. The formulas proposed by Karrer and coworkers [5d] for these two substances are as follows:



Beta carotene $C_{40}H_{56}$



Colorless vitamin A from liver oils, $C_{20}H_{30}O$.

It has been known for a long time that the empirical formula for carotene is $C_{40}H_{56}$. That the empirical formula for colorless A is $C_{20}H_{30}O$ (and not $C_{20}H_{32}O$) has been shown by Carr and Jewell [33] and more recently by Karrer and co-workers.[145] Recent work [145a] indicates that the chemical group which constitutes one-half the molecule of β-carotene is the specific precursor of vitamin A, and that the activity of other carotenes and carotenoid materials depends upon the presence of this radical in their molecules. Carotene and a number of other important plant and animal pigments have been discussed from the chemical point of view in Chapter IV, and it will be necessary here to give only some of the physiological aspects of the subject.

Interesting evidence as to how changes in chemical structure affect the physiological importance of organic compounds is to be gained from studies of the vitamin A activity of carotene and related compounds. Moore found that equal weights of β-carotene and colorless A had equal A activities,[195] while Euler, Karrer and Zubrys [88a] find the former one-half as active as the latter. On the other hand, β-xanthophyll, which, according to Karrer [144] differs from β-carotene only in the fact that one of the hydrogens in each of the terminal ionone rings is replaced by a hydroxyl group, is entirely inactive as a source of vitamin A, at any rate, for several species of animals.[152, 224] While there is some evidence that xanthophyll may serve as a source of A for guinea pigs [224] and pigeons,[88] this needs further confirmation.

There is already at hand a considerable body of evidence to show that colorless A does not occur in plant materials. Wolff, Overhoff, and von Eckelen [284] have used spectrophotometric and colorimetric methods to show that none of this material is present in carrots, green cabbage, spinach, or portulaca. Euler and others [82] find that the A activity of several other plant materials runs parallel to their carotene content. Morgan and Madsen [196] find that the A activity of apricots as determined by feeding experiments is, as nearly as can be determined, accounted for by their carotene content. Finally, Hartman and others [115] have carried out very careful experiments which show that the A activity of alfalfa hay as determined in feeding experiments with rats is entirely accounted for by its carotene content.

Moore [194] carried out experiments some time ago which indicated that cows converted carotene into colorless A within their bodies. This conclusion has been since confirmed by the work of Baumann and Steenbock [12] and of Gillam and others [97] which shows that when cows are fed

on such plant materials as pasture and artificially dried grass, which are rich in carotene but contain no colorless A, there is an increase not only in the carotene content, but also in the colorless A content of their butter.

Prior to 1930 the vitamin A values of food were determined as outlined above in feeding experiments with rats and given in terms of Sherman units. More recently, the carotene and colorless A contents of several foods have been determined spectrophotometrically. There has been much disagreement among different investigators as to the relative vitamin A activities of carotene and colorless A, and as to what quantity of either of these compounds would be equivalent to the Sherman unit.[12, 98, 97, 195] In recent careful experiments, however, Moore finds that equal weights of β-carotene and colorless A have equal A activities for rats when given in small doses;[195] while Euler, Karrer, and Zubrys[83a] find the former about half as active as the latter. This relation is undoubtedly influenced by a number of conditions[66a] and is different with different species of animals.[4a] From a purely theoretical consideration of the chemical structures of vitamin A and of β-carotene, Moore's findings seem possible; and from a consideration of the relative A activities of the various carotenes[159a, 83a] one might expect Moore's results where the physiological conditions for this conversion are most favorable.

Some idea of the A activity of 1 gamma of carotene or colorless A in terms of Sherman units can be gained from the work of Baumann and Steenbock[12] and of Gillam et al.[97] on butter. Baumann and Steenbock find that summer butter contains about 25 gamma of combined carotene and colorless A per gram; Gillam et al. find about 12 gamma. Summer butter, according to Fraps and Treichler,[93] contains about 50 Sherman units of A activity per gram. According to these figures, therefore, and on the supposition that carotene and colorless A have equal A activities, 1 gamma of either compound would have from 2 to 4 Sherman units of A activity. Recent careful work in the Bureau of Dairy Industry indicates that the A activity of β-carotene in terms of Sherman units lies within these limits.[115] It should be added, however, that this work was carried out with a basal ration which was very carefully devised to be complete in all nutritive essentials except vitamin A. It is probable that a good many A determinations have been carried out in the past with basal rations which were not complete in all essentials except A, and the average quantity of A or carotene required to produce a given rate of growth in past experiments is, therefore, probably greater than in these recent ones.

A standard crystalline preparation of β-carotene has recently been distributed by the League of Nations, and the international unit of A activity is defined as that given by 1 gamma of this preparation. Much further light will be thrown on this subject when different laboratories have had time to determine the A activity of this preparation according to their particular methods.

The vitamin A content of milk, butter and other foods. The factors which cause variation in the A content of foods may be divided into those which are physiological, and those which have to do with the

preparation of the food for consumption and with its storage. Two important physiological factors in the case of plants are heredity and the effects of admission or exclusion of sunlight during growth. Bills and McDonald have found that the carotene content of carrots varies all the way from 96 gamma per gram in the Early Scarlet Horn variety to 1.2 gamma per gram in the Isabell's Maude S. variety.[21] Kramer et al. find that the green outer leaves of lettuce, which have grown under the influence of sunlight, have 30 times as much vitamin A as the white inner leaves which have been shielded from that influence.[154] Fraps [98] gives interesting examples of the effects of drying and storage on the A content of foods. He found that carrots and spinach lost from 65 to 80 per cent of their A content as the result of drying, and that powdered whole milk and yellow corn lost from 30 to 80 per cent as the result of storage for several months.

In the case of milk, butter, and eggs, the studies which have been made so far indicate that the outstanding physiological factor which influences their A content is the A or the carotene content of the food of the animal which produces them. Thus Fraps reports [92] that the butter fat of cows fed for some months on cottonseed meal and cottonseed hulls contains only 2.5 Sherman units of vitamin A per gram, while that of cows on Sudan grass pasture contains 33 units per gram. The egg yolks of hens, which received through the winter a ration somewhat deficient in vitamin A decreased in their A content from 20 units per gram at the beginning of the winter to 5 units at the end.[249]

Vitamin A is fairly resistant to such heating as is likely to occur in cooking, provided that oxygen is excluded. The colorless A found in animal tissues is readily destroyed by heat in the presence of oxygen, while the carotene in some plant tissues has been found to be decidedly more resistant to this treatment.[245]

It is clear from the foregoing discussion that information in regard to the A content of foods does not lend itself readily to being put into a table. The A content of any given food may vary enormously according to how it is produced and how it is prepared for consumption. There are large differences also, however, in the original or maximum A contents of different foods. Table XCIX has been prepared to give an idea both of the differences which occur in individual foods, and of those which are found when different foods are compared with one another. For foods which contain moderate or high quantities of vitamin A, high and low figures have been given. These do not necessarily represent the extremes to be found in the literature, but give an idea of the range in experiments in which sufficient information is given for the purposes of the table. Unless otherwise stated in the table, it is to be assumed that the determinations were made on the raw foods. The figures given represent the A content of the fresh foods including their water content. In cases where the A content of the food has not been calculated in terms of Sherman units by the original authors from whom the data have been obtained, the figures for A content given in Table XCIX have been put in brackets.

For help given in compiling the table the authors are greatly indebted to the Bureau of Home Economics of the United States Department of Agriculture.

Table XCIX shows only how the A content of butter compares with that of a few other standard foods, which have been selected as being representative of widely different classes of common foods. Much further information on the subject is to be found in the text book of Sherman and Smith [245] and in recent articles by Fraps and Treichler [93] and by Rice and Munsell.[218]

Table XCIX.—The vitamin A content of representative foods as shown by feeding experiments with rats. For explanation, see text.

Food	Description	Sherman units per gram	Reference
Banana	Fresh	1.7	(68)
Banana	Fresh	3.5	(218)
Butterfat	From cows on feed low in vitamin A	2.5	(92)
Butterfat	From cows on pasture	50.0	(92)
Carrots	Fresh, yellow, young	32.9	(218)
Carrots	Fresh, yellow	67.0	(93)
Cod liver oil	Norwegian, finest non-freezing	[546.0]	(40)
Cod liver oil	Icelandic, unrefined	[4389.0]	(40)
Corn	Yellow, ground	2.5	(91)
Corn	Yellow, ground	8.0	(91)
Egg yolk	Boiled; from hens on food low in vitamin A	6.0	(249)
Egg yolk	Boiled; from hens on food high in vitamin A	30.0	(249)
Lemon juice		0.0	(218)
Lettuce	Head, chiefly white leaves	1.8	(218)
Lettuce	Green leaves	[21.3]	(66)
Liver	From animals on food low in vitamin A	5.0	(245)
Liver	Pig's	98.9	(218)
Meat	Average, beef muscle	0.2	(218)
Peas	Raw or canned	6.2	(218)
Peas	Green, raw, cooked, or canned	[20.0]	(67)
Spinach	Raw or canned	49.5	(218)
Spinach	Virginia Savoy, fresh	71.4	(150)
Turnips	White	0.2	(218)
Wheat	Whole	0.2	(93)

The factors which influence the A content of milk and butter have been discussed in Chapter IV and also in Chapter XV, and they need only be summarized here. Practically all of the vitamin A content of milk is associated with the fat, and the A content of milk can therefore be calculated from that of the fat if the fat content of the milk is known. In Table XCIX, therefore, figures for the A content of milk are omitted. The A content of butter is highly dependent on the carotene or A content of the food of the cow, and, as is shown by the figures in Table XCIX, may sometimes be twenty times as much as it is at other times.

As the food of cows consists usually entirely of vegetable materials, and as all of the A activity of vegetable materials is due to their carotene content, the A activity of butter, in so far as it is influenced by food, will be roughly parallel to the carotene content of the cow's food, and, there-

fore, to the natural yellow color of the butter. This parallelism between A activity and natural yellow color in butter will not hold where the butters of different breeds of cows are compared; but breed differences in the A content and natural yellow color of butter from cows on the same feed are small in comparison to those which can be brought about by changing the carotene content of the food.

Of the ordinary dairy feeds, fresh green plant material, such as is found in good pasture, has much the highest carotene content. Hay has a very variable carotene content; lower than that of good pasture, but higher than that of the grains and concentrates. Carrots have a high carotene content, but are not very largely used at present as a dairy feed.

The A content of butter and milk is, therefore, likely to be higher in summer when the cows are on pasture, than in the winter. But the carotene content of both pasture and hay is so variable that the A content of dairy products must be regarded as variable at all seasons.

Much of the butter sold in the winter is made from cream churned in the summer and kept in cold storage. The A content of butter kept in cold storage decreases slowly, and the A content of winter butter produced in this way is, therefore, likely to be higher than would be that of butter made from winter milk.

Vitamin B. Early knowledge of the B vitamins resulted from a study of the etiology of a human nervous disease known as beri-beri. In 1878, 30 per cent of the men in the Japanese navy suffered from beri-beri. In 1885, Takaki altered the diet of these sailors. Instead of polished rice with a little fish and vegetables, they were given wheat, barley, beans, milk, and meat with less rice. As a result beri-beri was quickly and practically completely eliminated. Since that time similar results on the incidence of beri-beri in various eastern countries have been obtained by making similar changes in the diet.

Since the discovery that beri-beri was a nutritional disease, it has been found by a number of investigators that milk contains, in addition to vitamin A, certain water-soluble factors which are necessary for the growth and well-being of animals, and of which very small quantities in the diet produce remarkable physiological effects. The growth-promoting material present in milk acts to some extent as a cure for beri-beri, and, for some time, the name vitamin B was given to a group of water-soluble compounds, all of which are present in milk and in yeast, but which were always recognized as being distinct from the anti-scorbutic water soluble vitamin C. In time, however, it has become more and more clear that what was called vitamin B is really a group of several nutritionally important compounds. With regard to two of these much knowledge has already been accumulated. There is no doubt that several others exist, but our knowledge of these is still very scanty. For a much fuller account of this subject and of all the vitamins, the reader is referred to the textbook of Sherman and Smith.[245] References to the literature cited in the immediately following discussion are also to be found in this textbook.

In 1926 Smith and Hendrick reported an experiment that showed con-

vincingly that what had been known as vitamin B was composed of at least two substances that were necessary to prevent polyneuritis and promote normal growth in rats. Seidell had prepared a picrate of the antineuritic vitamin. It had been tested with pigeons and found to be very potent as a source of the antineuritic vitamin; but, when Smith and Hendrick fed it to rats in quantities which they judged sufficient, considering this potency, it failed to induce growth. When autoclaved yeast was similarly fed, the rats lost weight and died. When the picrate and autoclaved yeast, which was known to be inactive in the treatment of polyneuritis, were fed together, good growth occurred. Seidell obtained similar results with rats, and further found that the autoclaved yeast did not promote the growth of pigeons that were protected from polyneuritis by means of his vitamin preparation. During the same year Goldberger, Wheeler, Lillie and Rogers suggested that a heat stable substance in yeast was concerned with the prevention and cure of pellagra. In 1925 Goldberger and Tanner had announced that a "heretofore unrecognized or unappreciated dietary factor, which we designate as factor P-P," is concerned in the prevention (and presumably the cause) of this disease. The factor, they concluded, is present in brewers' yeast, milk and lean beef. Goldberger and co-workers had studied the disease known as "black tongue" in dogs and considered it the analog of pellagra. They were able to produce it in dogs by feeding diets similar to those used by people suffering from pellagra, and found yeast, yeast concentrates, and autoclaved yeast very active in curing this disease in dogs. They also worked with rats. To the ration of rats on a vitamin B free diet, they added autoclaved yeast. The rats ultimately failed to grow, some developing symptoms of polyneuritis. When, instead of autoclaved yeast, they used an alcoholic extract of corn meal, the rats likewise ultimately failed to grow, although the extract was capable of curing polyneuritis. When the autoclaved yeast and alcoholic extract of corn were both used, the rats grew normally. Lean beef behaved similarly to the autoclaved yeast. When, as a source of vitamin B, their rats were fed a small amount of one of Seidell's concentrated vitamin preparations, the rats declined in weight without evidence of polyneuritis; but in some cases developed symptoms suggestive of pellagra. On the further addition of autoclaved yeast the rats resumed growth and recovered from the "pellagra-like" symptoms. Goldberger and co-workers came to the conclusion that autoclaved yeast and beef muscle contain a factor distinct from the polyneuritis-preventing vitamin which in combination with it is essential for the growth of the rat. "From the facts presented," they say, "it seems probable that this is the same as the factor P-P."

The Biochemical Society in England has adopted the name vitamin B_1 for the more heat-labile (when autoclaved), antineuritic factor discovered by Eijkman in 1897; and vitamin B_2 for the more heat stable (when autoclaved) factor discovered by Goldberger and others in 1926, and found necessary for the prevention of dermatitis in rats. Sherman suggested

the terms vitamin F and vitamin G, respectively, for these factors. The term vitamin B is now used frequently to refer to vitamin B_1, or F.

From this point on the discussion will deal with work which has appeared since the latest addition of Sherman and Smith's textbook. Some of the articles will be cited directly; references to the others will be found in Volume III of the Annual Review of Biochemistry.[7]

Numerous efforts have been made to isolate vitamin B_1 in pure crystalline form; and up to the present time several laboratories have prepared potent crystalline preparations which are very similar in crystalline structure, are of about the same order of potency, and resemble each other closely in chemical analysis. The preparations from different sources (yeast and rice bran) are identical. The analyses of some of these preparations are expressed by the following formulas:

$C_{12}H_{16}N_4O_2S.2HCl$ (Made by Ohdake)
$C_{12}H_{16}N_4OS$. (Made by Windaus and co-workers)
$C_{12}H_{18}N_4O_2S.2HCl$ (Made by Jansen and co-workers)

Kinnersley, O'Brien and Peters have made crystalline preparations that are fully as active or more active than those above; as little as 2.17 gamma per day being required to cure and protect a pigeon from polyneuritis. They conclude, "Neither ourselves nor others have yet reached pure vitamin B_1." Seidell and Smith have recently described a more direct and improved method of preparing crystalline vitamin B_1 of about the same potency as the preparations of Jansen, Windaus and Peters.

Block and Cowgill described a method for purifying vitamin B_1, based on the theory that it is an organic base and as such should be capable of extraction from aqueous alkaline solutions by organic solvents. They used aqueous extracts of rice polishings and of yeast and obtained their best results when these aqueous extracts were adjusted to about pH 9 or 10. Considerable concentration of the vitamin was then obtained by extracting with a number of organic solvents. Much of their work was done with the use of ethyl ether. The extraction was carried out at reduced temperatures. Eighty to 90 per cent of the total vitamin potency was recovered from the organic solvent; the impurities, based on total solids, were reduced about 500 times; while the nitrogenous impurities were reduced 200 times. They believed that about 10 per cent of the nitrogen in this concentrate was vitamin nitrogen.

Seidell[281] has studied the extraction of vitamin B_1 from yeast by percolation with various concentrations of methyl alcohol, ethyl alcohol, and of acetone, and the effect of acidity on this process. He found a 70 per cent concentration of any of these solvents, acidified with 1 per cent HCl, to be most advantageous. With 70 per cent ethyl alcohol, 80 per cent of the vitamin B_1 was extracted and only 10 per cent of the yeast solids. Smith[252] reports a similar study in which he finds that 76 per cent ethyl alcohol and 70 per cent acetone plus 1 per cent of HCl, may be used to remove 80 per cent or more of vitamin B_1 in dried brewers' yeast without removing appreciable quantities of vitamin B_2. Stiebeling and Alle-

man [257] have tried extracting these vitamins from skim milk powder using 80 per cent ethyl alcohol either alone or rendered 0.1*M* with gallic, benzoic or hydrochloric acid. The vitamins B_1 and B_2 in the extract and in the residue, were determined. With B_1 they obtained their best results with the plain 80 per cent alcohol; 60 per cent of the vitamin originally present in the skim milk powder was extracted and 40 per cent remained in the residue. Only 10 per cent of the vitamin B_2 was found in this extract. With vitamin B_2 only 40 to 45 per cent of that originally present could be accounted for by that in the extract plus that in the residue, except with gallic acid. In this case 30 per cent of that originally present was recovered in the extract and 35 per cent in the residue. There was evidently a considerable destruction of this vitamin.

Bourquin and Sherman [25] describe a method of making a crude preparation of vitamin B_1 from wheat by extracting with 80 per cent alcohol. Wheat germ meal has been similarly used. In some cases the extracted vitamin is subsequently brought into aqueous solution, the solution treated with lead acetate, the lead removed, the vitamin adsorbed from the solution onto fuller's earth, and fed in this combination. From the work on flavines it would appear that the Bourquin preparation is deficient in some B factor other than vitamins B_1 and B_2. Kinnersley, O'Brien, Peters and Reader describe a large scale method of preparing vitamin B_1.

A number of adsorbing reagents have been used in concentrating the B vitamins, e.g., fuller's earth,[229, 230] Norite charcoal,[211] and silica gel.[165] The extent of the adsorption from a solution depends upon its pH; and vitamins B_1 and B_2 are adsorbed to a different extent by the same adsorption reagent at the same pH. These facts have been used in the purification and separation of these vitamins.

Chapter IV describes the methods of isolation and properties of lactoflavine (formerly called lactochrome), the pigment in the whey of milk. It has been isolated in crystalline form and is of the composition expressed by the formula $C_{16}H_{20}N_4O_6$ or $C_{17}H_{20}N_4O_6$. When fed to rats which have been depleted on a diet free of vitamin B_2 (but containing vitamins B_1 and B_4), the animals resume growth. On as little as 5 gamma per day of this crystalline preparation, rats gained 35 to 75 grams per week. Lactoflavine appears to be identical with the flavines isolated from other sources (e.g., ovoflavine) which are also physiologically active. These flavines may be identical with vitamin B_2 or an important constituent of this factor.

Warburg and Wieland have isolated a yellowish-red oxidation ferment from yeast and from lactic acid bacteria. It has a high molecular weight, and appears to be a combination of a flavine with a colloid group. Besides functioning as an enzyme, this combination has the same action in the diet as vitamin B_2. It can be split into a flavine, which is also active as vitamin B_2, and a colloidal substance; but it then loses its enzyme activity.

Booher [24] has recently described a method of concentrating lactoflavine, thus obtaining a potent vitamin B_2 preparation from whey.

In the assay of vitamin B_1, vitamin B_2 is generally supplied by the use of autoclaved yeast.

It should not be assumed from the way in which the terms vitamin B_1 and B_2 were defined above that it is known definitely that what is now called the antineuritic vitamin is a single substance or that the same is true of the pellagra-preventive vitamin; nor do we know that all of the physiologically active substances formerly included in the vitamin B complex are either antineuritic or pellagra-preventive.

In 1929, Reader brought forth evidence which she took to indicate that in addition to vitamin B_1, the crude watery or alcoholic extracts of yeast contain another heat- and alkali-labile factor necessary in the nutrition of the rat. (See Barnes, O'Brien, and Reader [9] for reference.) She described a method of preparing this factor free of vitamin B_1. It is present, according to Kinnersley, O'Brien and Peters, in the concentrated crystalline preparations that they have made of vitamin B_1; and according to Harris [7a] it is present in very small quantities in all crystalline preparations of vitamin B_1 that have been made. Reader describes the symptoms of rats suffering from a deficiency of this factor. The rats show a lack of muscular coordination, inability to walk or normally to control the movements of their heads,—symptoms associated generally with polyneuritis. She and coworkers designated this new factor vitamin B_4 (previously B_3) and gave a method of assaying it and of preparing it in concentrated form. A crystalline preparation which they made had a composition agreeing with the formula $C_4H_4N_4.HCl.\frac{1}{2}H_2O$. Tschesche showed that these crystals were mainly adenine hydrochloride. This was confirmed by Bernal and Crowfoot. Heard, Kinnersley, O'Brien, Peters and Reader, however, tested adenine hydrochloride and found it inactive as a source of B_4. It has been suggested that these crystalline preparations of vitamin B_4 are mainly adenine hydrochloride, the vitamin B_4 being present as an impurity. The actual existence of some physiologically active factor in these crystalline preparations is strikingly shown independently by the work of György, Kuhn and Wagner-Jauregg. They added lactoflavine or ovoflavine to the ration of rats on the Bourquin-Sherman diet, which contained an alcoholic extract of ground whole wheat as its source of vitamin B_1. Even 50 gamma of the flavine produced no response; but the flavine was active as a source of B_2 when the rat was in addition supplied with a crystalline preparation of vitamin B_4. Adenine could not be substituted for this B_4 preparation.

Evidence also exists that autoclaved yeast contains, in addition to vitamin B_2, another substance that is even more stable toward heat and alkalis, and which is required for the normal nutrition of the rat. Chick and Roscoe [36] showed that egg-white, while a relatively good source of the antidermatitis vitamin B_2, is lacking in B_1. Chick and Copping [37] fed egg-white and concentrates made from it to rats as a source of vitamin B_2. Their basal diet contained vitamin B_1 and presumably B_4. Sustained growth did not occur; but "satisfactory development over long periods, including fertility and successful pregnancy, was attained" when, instead

of egg-white (or egg-white concentrate) to supply vitamin B_2, autoclaved yeast or autoclaved watery yeast extracts were used. They call the "hitherto unrecognized dietary factor," which withstands prolonged autoclaving in alkaline solution and occurs in autoclaved yeast along with vitamin B_2, factor Y. Not much is known regarding this factor or how it was supplied when the flavine from egg-white produced growth when fed along with a concentrate of B_1 and the crystalline preparation of B_4. It has been found to occur in green leafy vegetables, egg-yolk and ox-liver; but is deficient in wheat embryo, meat (ox-muscle), etiolated leaves of green vegetables and onions as well as in egg-white.

The growth-promoting and antidermatitis action of the more heat-stable fraction of the vitamin B complex is frequently referred to as one and the same factor; but that this is true has been questioned and never has been proved.[252] References, bearing on this question, are given in two recent papers by Roscoe, in the latter of which she shows that the potencies of several preparations, which she tested for their capacity to cure dermatitis and to promote growth of rats, run parallel. She obtained no support for the theory that two separate dietary factors exist, one responsible for the prevention and cure of dermatitis and the other for the promotion of growth of the rat.

Vitamins B_1, B_2, B_4 and factor Y are considered to be necessary for the normal nutrition of the rat. The pigeon is thought to require vitamin B_1 and apparently one or two other factors that were originally included in the vitamin B complex. Williams and Waterman [279] have described a substance called vitamin B_3 which they considered necessary for the pigeon. Its existence as a factor distinct from vitamin B_1 is questioned by a number of workers.[7b] Carter, Kinnersley and Peters also describe a factor called vitamin B_5 that they claim is required by pigeons.[84]

According to Harris [5b] "something like a score of authors claim to have demonstrated the existence of new factors having mostly a distribution similar to vitamin B" and included originally in the vitamin B complex. Many of these factors it seems may finally be found to be identical. That what was originally called vitamin B is rather complex, is certain. That it consists of at least one heat-labile and one relatively more heat-stable factor, when subjected to autoclaving, there is no doubt; but when it is attempted to differentiate between various heat-stable and heat-labile constituents, our knowledge at present is very uncertain and confusing.

Although vitamin B_2 is not required by the pigeon, Kline, Keenan, Elvehjem and Hart find chicks do need it.

The bacterial synthesis of vitamin B in the mammalian intestinal tract. The work on the discovery of the B vitamins demonstrated at the same time that in general the animal organism can not itself synthesize these factors and that they must be supplied in the food. They can be synthesized by plants, including many species of yeasts and bacteria. With rats on a diet that does not contain significant amounts of vitamin B complex, the feces may contain considerable quantities of it. Occasionally rats on such a diet or on a diet lacking only in vitamin B_1 or B_2, may

recover spontaneously from the deficiency symptoms, grow normally to maturity and reproduce. This phenomenon has been called refection. It is very likely due to the bacterial synthesis of adequate amounts of the B vitamins in the intestinal tract of these animals.[1871]

Theiler, Green and Viljoen[259] fed rations very low in the vitamin B complex to cattle, and from their experience suggested that the cow may synthesize these vitamins. Bechdel and co-workers showed that calves may grow normally to maturity, produce normal off-spring and secrete milk of normal vitamin B content for a few weeks on a ration that carries an insufficient amount of the B complex to support growth and well-being in rats.[17] To explain this result they carried out an experiment which demonstrated the synthesis of these vitamins in feed obtained from the rumen of the cow. They isolated bacteria from the rumen, developed cultures on a nutrient agar medium (which was tested with the rat and found free of the vitamin B complex), brought the cultures to dryness, and fed this material to rats as their only source of the B vitamins. The rats grew about as well as on an equal weight of yeast. These workers also took wet fermented feed from the rumen of a cow that was receiving a ration practically devoid of the B complex when tested on rats. This material from the rumen was allowed to ferment at 37° for five days. It was then extracted and concentrated so that one gram of the final preparation contained the B vitamins, as far as possible, that may have been present in 25.4 grams of the original fermented rumen material. This preparation was fed to rats as 50 per cent of their ration to supply the vitamin B complex. The rats, which had ceased to grow because of this deficiency, resumed growth and grew constantly at a subnormal rate throughout an experimental period of 8 weeks. It is likely that this growth was greater than could be accounted for by the concentration of the very small amounts of the B vitamins in the cow's feed. The authors conclude that the vitamin B complex is produced in the rumen of the cow by bacterial fermentation, and that this synthesis offers a satisfactory explanation of the growth, reproduction and inception of lactation with their heifers on rations too low in their content of the vitamin B complex to promote growth and well-being in rats. This experiment of Bechdel and co-workers was carried out when little was known about the complexity of what was then called vitamin B. The rat tests indicated the deficiency in the cattle rations of some essential component of this complex, but not of all of its constituents. The commercial casein in these cattle rations most likely carried considerable quantities of vitamin B_2; and it must be borne in mind that these rations were inadequate in some respect to support continued lactation. This fermentative synthesis of the B vitamins is apparently more general and more active in cattle than refection in rats which occurs only occasionally, appears to be transmitted from rat to rat, to be entirely prevented by autoclaving the feed, and to be associated with an abnormal condition in the digestion of starch. Little is known about the conditions that may influence this fermentative synthesis in cattle, the source of the bacterial infection, the effect of various treat-

ments of the feed, or how generally adequate it may be to supply the needs of the animal under all circumstances. Until further light is available one may hold from these experiments of Bechdel that the fermentative process in the intestinal tract of young cattle may furnish enough vitamin B_1 for normal growth and reproduction and may or may not furnish enough vitamin B_2 for these functions. With animals other than the cow this synthesis of the B vitamins appears in general to be inconsiderable, and the animal to be dependent upon its diet for these factors.

Methods of estimating the B vitamins; definition of units. There are no satisfactory chemical methods for the quantitative determination of any of the B vitamins. Biological methods have been developed for the assay of vitamins B_1 and B_2. With vitamin B_1 both birds (pigeons, fowls, etc.) and rats have been used. When birds are used, the quantity of food or preparation necessary to furnish enough vitamin B_1 to protect the fowl from polyneuritis or to maintain its weight or to cure it after it has developed polyneuritis on a vitamin B_1-free ration have been taken as a measure of the potency of this food or preparation. The curative procedure, however, seems to be preferred by most workers who use birds; and either the duration of the cure following the administration of a given dose of the material in question or the percentage of the birds cured furnish the results for the evaluation of the potency of the material in question. These procedures have recently been very carefully investigated by Coward and co-workers [41] and their relative merits fully discussed. References will be found in this article or in Vol. III, Annual Review of Biochemistry,[7] to the work of the investigators quoted below.

When rats are used to determine vitamin B_1, the most common procedure is to take the young at weaning, deplete their stores of vitamin B_1 by feeding a diet that is adequate in every other respect, then add varying quantities of the material in question, and take the growth that is thereby produced as a measure of its potency. Rats frequently do not develop polyneuritis on a diet containing very little vitamin B_1; and, in the procedure just outlined, the failure of the rat to grow is taken to indicate the end of the depletion period. The daily quantity that is required of the material in question to produce an average growth of 3 grams per week for a period of 4 or 8 weeks is taken as containing one Sherman unit of vitamin B_1. Roscoe, and Aykroyd and Roscoe use as their standard of comparison, the quantity of material necessary to produce "normal growth" of the rat, i.e. an average growth of 50 to 60 grams in 5 weeks. Coward and co-workers (above) have described a procedure involving a recovery period of only 3 weeks.

The Health Organization of the League of Nations has adopted a certain preparation of this vitamin as an "International Standard Preparation," which is issued with a descriptive pamphlet and described therein as "a concentrated preparation of the anti-neuritic vitamin (B_1), adsorbed on kaolin from a watery extract of rice polishings at a reaction adjusted to pH 4.5, and subsequently dried. The preparation was carried out in the Medical Laboratory, Batavia, Java, by the method of Seidell as de-

scribed by Jansen and Donath (1927)." An "International unit has been defined as the anti-neuritic activity of 10 milligrams of the International Standard Preparation."

"The curative dose for a pigeon of 300 grams weight, suffering from polyneuritis, on a diet of polished rice, is about 0.02 to 0.03 gram (20 to 30 mgs.) of the preparation per diem, following the method of Peters and Kinnersley."

Table C.—The vitamin B_1 content of various foodstuffs.
(Except as indicated in the footnotes the values are taken from Roscoe (1931), and are based on rat growth experiments.)

Material	Daily dose needed to promote an increase in rat weight of 50-60 grams in 5 weeks	Relative potency. Dried brewers' yeast = 100	
	Grams (dry wt.)	Dry weight basis	
Yeast (dried)	0.05	100	
Milk (cow's)*	1.25-2.5	2-4	Milk 2-4
Milk (human)**	>2.5	<2	
Wheat flour (white)†		nil	Cereal products 0-50
Wheat germ	0.10	50	
Apple	1.0-2.0	2.5-5	Fruits 2.5-20
Banana	1.0	5.0	
Orange	0.25	20	
Tomato	0.5-1.0	5-10	
Carrot	0.25-0.5	10-20	Roots 2.5-20
Onion	0.5-1.0	5-10	
Potato	1.0-2.0	2.5-5	
Turnip	0.5-1.0	5-10	
Cabbage (etiolated)	0.3-0.5	10-17	Leafy foods 5-17
Cabbage (green)	<0.95	>5	
Lettuce	0.3	17	
Spinach	<0.5	>10	
Watercress	<0.3	>17	
Egg white		nil	From Animal Sources 0-10
Egg yolk	0.5-1.0	5-10	
Liver	<0.5	>10	
Meat (ox muscle)	1.0-1.5	3-5	

* From Outhouse et al. (1927)[208] and Hunt and Krauss (1931).[138]
** From Donelson and Macy (1934).[57]
† From Medical Research Council Monograph.[187]

"The daily dose of the preparation required to maintain normal growth in a young rat of 50 to 100 grams weight, on a diet which is complete in all other respects, and which includes the anti-dermatitis vitamin (B_2), is about 0.01 to 0.02 gram (10 to 20 mgs.)."

The curative or preventive method of determining vitamin B_1 is more specific than the rat growth method; but, according to Coward and co-workers, the probable error of the pigeon curative method is about five times that of the rat growth method when the same number (nine) of test animals is used in each case. Calculated in terms of International Units, the results obtained by these workers, by the two methods, were not concordant.

Vitamin B_2 is determined by the rat-growth method with a procedure

analogous to that used with vitamin B_1, except that the rats are first depleted of vitamin B_2 by feeding a ration deficient only in this respect. The Sherman unit for vitamin B_2 is analogous to that for vitamin B_1; and a "normal growth" of 50 to 60 grams in 5 weeks is also used as a standard of growth in the measurement of this vitamin. Hartley [114] has

Table CI.—The vitamin B_2 content of various foodstuffs.

(Except as indicated in the footnote the values are taken from Roscoe (1931) or from work that was done in the same laboratory and is referred to in this reference. The results are based on rat growth experiments.)

Material	Daily dose needed to promote an increase in rat weight of 50-60 grams in 5 weeks	Relative potency. Dried brewers' yeast = 100	
	Grams (dry wt.)	Dry weight basis	
Yeast (dried)	0.1	100	
Milk (cow's)	0.7	14	Milk 6-14
Milk (human)*	>1.6	<6	
Egg white	0.3-0.6	17-33	Foods from animal sources 10-83
Egg yolk	1.0	10	
Liver (ox)	0.12	83	
Meat (ox muscle)	0.5-0.75	14-20	
Cabbage (etiolated)	0.4-0.8	12-25	Leafy foods 12-25
Cabbage (green)	0.4	25	
Lettuce	0.6	17	
Spinach	0.4	25	
Watercress	0.4	25	
Carrot	0.5-1.0	10-20	Roots 2.5-20
Onion	1.6	6	
Potato	2.0-4.0	2.5-5	
Turnip	1.0	10	
Apple	2.0-4.0	2.5-5	Fruits 2.5-10
Banana	1.0-2.0	5-10	
Orange	1.0-2.0	5-10	
Tomato	<2.0	>5	
Peas (dried)	2.0-3.0	3-5	
Maize (whole white)	2.0-3.5	3-5	Cereals and cereal products 0-10
Maize (whole yellow)	>4.0	<2.5	
Maize endosperm ("grits" or "polenta")		Very low	
Millet	>4.0	<2.5	
Rice	>4.0	<2.5	
Rice endosperm		Very low	
Wheat (whole)	2.0-3.0	3-5	
Wheat endosperm ("Patent" or "household" flour)		Very low	
Wheat germ	1.0-2.0	5-10	

* Donelson and Macy (1934).[57]

published a dissertation on "Factors concerned in the quantitative determination of vitamins B (B_1) and G (B_2)" which deals with the rat growth procedure for the determination of these vitamins.

The amounts of vitamins B_1 and B_2 in milk and other foods are shown in Tables C and CI. All of these figures are based on rat growth experiments. The B_1 content of a number of foods has been determined in experiments with pigeons by Plimmer et al. (1933). In most cases the

results with pigeons agree fairly well with those obtained with rats, but there are a number of notable exceptions.

Milk as a source of the B vitamins. Cow's milk contains all of the B vitamins that are necessary for the normal nutrition of the rat. This is attested by the fact that these animals may go through several generations with no other source of these factors in their diet.

Numerous determinations have been made of the potency of the vitamin B complex as a whole in milk using the rat as a test animal. This work is reviewed by Gunderson and Steenbock [99] where the references that are not given here will be found. The technique has varied in this work from time to time and in different laboratories; the strains of rats that different workers have used have varied; and the results are hard to compare and are frequently conflicting. The work with rats up to 1926, when vitamin B_2 was discovered, may be summed up as follows:

(1) The quantity of cow's milk reported as necessary to supply enough of this complex to promote approximately normal growth varies from 2 cc. (Hopkins) to 16 cc. (Osborne and Mendel).

(2) While Hopkins found milk from goats on "summer" and "winter" rations equally potent as a source of this complex, and Osborne and Mendel found cow's milk equally rich in this factor in summer and winter, Kennedy and Dutcher found cow's milk to vary in potency depending on the diet of the cow. Their best milk was about 50 per cent more potent than their poorest. Ten cc. of the former, they found, supplied enough of the vitamin B complex to promote normal rat growth.

Since this work of Kennedy and Dutcher, Bechdel and Honeywell (1927) and Gunderson and Steenbock (referred to above) have studied the effect of various conditions upon the potency of cow's milk as a source of the vitamin B complex. Bechdel and Honeywell tested the milk from cows that for two years or more had been fed a ration so poor in the vitamin B complex that it failed to supply enough of this factor to support growth and well-being in rats. Twelve cc. of this milk per day per rat supported normal growth. No better growth was obtained with 16 cc. or 20 cc., and milk from cows on normal rations was no more potent in this respect.

Gunderson and Steenbock compared milk from cows of different breeds (Durham, Guernsey, Holstein), from cows on ordinarily good rations and on rations especially rich in their content of the B vitamins, and from cows at different stages in lactation. None of the factors that they investigated seemed to affect materially the potency of the vitamin B complex in the milk. They compared the milk from goats on an ordinarily good ration with that from goats receiving about two-thirds more of the vitamin B complex. The concentrations of the vitamin B complex in these milks were practically the same, and very close to that of the cow's milk with which they were working. With neither the cow's nor goat's milk was 12 cc. sufficient as a source of the vitamin B complex to promote normal rat growth.

Macy and co-workers, depleting their rats and using all the modern

refinements of technique, have made very careful determinations of the vitamin B complex in cow and human milks. Outhouse, Macy, Brekke and Graham[208] found that rats that had been taken at weaning and depleted of their stores of the B vitamins, grew normally for four weeks when given 12 cc. of certified cow's milk daily to supply the B vitamins; 16 cc. daily produced normal growth for eight weeks; while 20 and 25 cc. produced excellent growth, average or above, for about 12 weeks, followed in a number of instances by cessation of growth. The addition of 0.4 gram daily of yeast to the diet of these rats brought about an immediate resumption of growth. Macy, Outhouse, Graham and Long (1927) found that 16, 18 or 20 cc. of human milk, when similarly fed to rats as a source of the vitamin B complex, permitted less than average growth. Sexual maturity was delayed in the females on levels below 18 cc.; normal ovulation occurred on a minimum of 20 cc.; reproduction occurred on a minimum of 25 cc.; 25 and 30 cc. daily were required to supply enough of this complex for normal growth and sexual activity in the young; "while 35 cc. were necessary to produce continuous growth in larger rats weighing 200 to 240 grams." Even with 35 cc. daily of human milk, mother rats were unable to rear their young, the milk being either qualitatively or quantitatively insufficient. Growth with 30 and 35 cc. daily of human milk, was not as satisfactory as with 20 cc. of cow's milk when fed to rats as the source of their B vitamins. However, these workers report that the rats receiving over 14 cc. daily of human milk in this experiment suffered from diarrhea. It must also be borne in mind that the very large quantities of milk fed would greatly reduce the consumption of the basal ration and that other deficiencies might thereby become effective.

Donath[55] has reviewed the work up to 1929 in which birds were used to determine the antineuritic vitamin (vitamin B_1) in milk, and the references to this work will be found in his paper. The work reviewed indicates that neither cow nor human milk is a rich source of this vitamin. Mothers on diets poor in this factor may develop beri-beri soon after parturition, and their babies may develop infantile beri-beri. The milk of these mothers may be insufficient in quantity; but there is some evidence that it is also poor in its concentration of the antineuritic vitamin. Infantile beri-beri is frequent in certain portions of the world, but not in Europe or America.

The situation with respect to the antipellagric factor (vitamin B_2) in milk is quite different from that with vitamin B_1. Milk was early found to be effective in the treatment of pellagra (Casal, two centuries ago; and Roussel, eighty years ago); and Goldberger and co-workers, in experiments with rats and dogs as well as with human beings, found it an excellent source of vitamin B_2.

In accordance with these observations are those of a number of workers to the effect that both cow and human milk are relatively poorer sources of vitamin B_1 than of vitamin B_2 when used to supply these factors to growing rats. As a result of the work of Sherman and Axtmayer,[243] Hunt and Krauss,[187] Macy and co-workers,[208] and Samuels

and Koch,[225] it has been shown that substances relatively rich in vitamin B_1 but poor or practically devoid of vitamin B_2 (such as wheat, an alcoholic extract of wheat, or a crude preparation of vitamin B_1 that was made according to the method of Kinnersley and Peters) supplement cow's milk as a source of the B vitamins; and that autoclaved yeast, which is rich in vitamin B_2 but practically devoid of B_1, does not do so.

Quantitative determinations have also been made of vitamins B_1 and B_2 in both cow and human milks; and the influence of the diet upon these factors has been studied in both cases. Hunt and Krauss [137] found that 5 cc. of cow's milk supplied enough vitamin B_2 for normal rat growth; but they obtained better growth with 10 cc. They later [138] compared the concentrations of vitamins B_1 and B_2 in milk from cows on a good ordinary "dry" ration with that from cows on a similar ration fed along with pasturage. There was practically no difference in the vitamin B_1 content of these milks; whereas milk from cows receiving pasturage was 50 to 75 per cent more potent as a source of vitamin B_2 than that from cows that received only the dry ration. They also obtained a very striking correlation between the concentration of vitamin B_2 in the pasture grass and that in the milk at different times during the feeding of pasturage. The milk studied by Hunt and Krauss may be considered to contain about 0.15 Sherman unit of vitamin B_1 per cc. and possibly 0.4 to 0.6 or 0.7 Sherman unit of vitamin B_2.

MacLeod, Brodie, and Macloon [181] determined the potency of vitamins B_1 and B_2 at different seasons of the year in milk from a dairy of stall-fed cows. The cows were fed alfalfa hay of excellent quality, corn silage and a concentrate. The authors conclude that the variations that they found throughout the year in the concentration of these vitamins were small and probably not significant. The milk contained about 0.1 Sherman unit of vitamin B_1 and 0.3 Sherman unit of vitamin B_2 per cc.

Samuels and Koch [225] determined the quantities of vitamins B_1 and B_2 in cow's milk. They found that 25 cc. supplied enough of the former and 17 or 20 cc. enough of the latter to promote optimum growth of the rat. Compared at levels at which growth would be more sensitive to variations in the quantities of these factors that were fed, their samples of milk were only about one-half as potent as a source of vitamin B_1 as of vitamin B_2. If the concentration of vitamin B_1 be expressed as 0.25 to 0.3, that for vitamin B_2 might be taken as 0.5 to 0.6 Sherman unit per cc. They also determined the quantity of milk necessary to supply enough vitamin B_1 for successful lactation with the rat. According to their results, it was about 50 cc. daily. A number of workers have found the vitamin B_1 requirement for lactation to be 3 to 5 times that for normal growth.

As shown in the table giving the vitamin B_2 content of foods, Roscoe found that the quantity of milk equivalent to 0.7 gram of dry matter (i.e., 5 or 6 cc.) furnished enough of this factor to promote "normal" rat growth (i.e., 50 to 60 grams in 5 weeks with young rats). This would possibly correspond to a potency of about 0.7 Sherman unit per cc.

Dutcher, Guerrant and McKelvey [65] in a recent paper, have obtained

a similar potency for vitamin B_2 in cow's milk; but, surprisingly, when they tested the same milk for vitamin B_1, they found the potency of this vitamin to be very little less than that of vitamin B_2.

Donelson and Macy [56, 57] have recently published two papers on the vitamin B factors in human milk. In the first paper, they found that 20 cc. of this milk did not supply enough vitamin B_2 to promote optimum rat growth. They express the potency of this factor as about 0.2 to 0.3 Sherman unit per cc. In the second paper they studied the effect of diet upon the concentrations of vitamins B_1 and B_2 in human milk. The milk was analyzed when the mothers were on a diet that would be considered adequate according to ordinary standards, and again when these mothers were consuming 10 grams of yeast daily in addition to their ordinary ration.

These workers found no difference in the vitamin B_1 potency of these milks; but vitamin B_2 was about 50 per cent more potent in the milk secreted during the period when yeast was included in the diet. One cc. of either milk contained about 0.1 Sherman unit of vitamin B_1; and about 0.2 and 0.3 Sherman unit respectively of vitamin B_2. These results on the effect of diet on the concentration of these factors in human milk are very similar to those noted above with cow's milk.

One may sum up the work that has been done on the B vitamins in milk, as follows:

1. It would seem to indicate that milk is not a particularly rich source of vitamin B_1. It may take 20 or 25 cc. of cow's milk to supply enough of this factor for continuous normal growth, and 50 cc. for the successful lactation of the rat. Goat's milk is very similar to cow's milk in this respect; and human milk on the average may be somewhat less potent.

It may be noted that, whereas Donelson and Macy found 0.1 Sherman unit of vitamin B_1 and 0.2 to 0.3 Sherman unit of vitamin B_2 in human milk, the figures for the former in cow's milk vary in general with different investigators from 0.1 to 0.3 Sherman unit and for the latter from 0.3 to possibly 0.7 Sherman unit per cc. With cow's milk, 5 to 10 cc. as a source of vitamin B_2 may often produce very good growth in rats; but there probably is a very wide range between the "necessary" and "optimum" requirements of this vitamin (Sherman and Ellis [248]).

2. The concentration of vitamin B_1 in cow's, goat's and human milk is not readily increased above certain maximum limits; and in so far as diet is concerned, these limits are reached on rations that are ordinarily used and considered adequate. In regard to the possibility of reducing the vitamin B_1 content of cow's milk, the evidence is somewhat contradictory, but tends, on the whole, to indicate that it cannot be appreciably reduced. On the other hand, there is considerable evidence that would tend to indicate that with human and rat's milks the concentration of vitamin B_1 is reduced by continued feeding of rations that are inadequate in this respect. It seems quite likely that vitamin B_1 is not produced by bacterial activity in the human intestinal tract as it is in that of the cow.

The concentration of vitamin B_2 in cow's milk and human milk seems

to be more readily altered by dietary changes than that of vitamin B_1; but the increases that may be brought about by feeding materials especially rich in vitamin B_2 as compared with those ordinarily used, is not great (possibly 50 to 75 per cent).

3. Milk, being relatively richer in vitamin B_2 than in vitamin B_1, supplements and may readily be supplemented by the cereals as a source of the B vitamins.

In those countries where polished rice makes up a large portion of the diet and where beri-beri is still prevalent, infantile beri-beri is very frequent. Until recently, however, most investigators were of the opinion that practically no evidence existed of a deficiency of vitamin B_1 in infant feeding in this country or in Europe; but of late a number of clinical observations with children have been published in this country in which the authors claim that cow's and human milk may, more often than hitherto suspected, fail to supply infants with enough of this factor for optimum growth and well-being. References to this work are given in the paper by Donelson and Macy (1934) referred to above.

Effect of heating on the B vitamins in milk and milk products. Vitamin B_1 is often referred to as the heat-labile and vitamin B_2 as the heat-stable B vitamin, due to the difference in their rates of destruction when heated moist in an autoclave. However, vitamin B_2 does not completely escape destruction. The rates of decomposition of these vitamins depend upon a number of factors. Much of the work has been done with impure mixtures. Sherman and Grose [236] found that when tomato juice was heated at pH 4.38, an increase of 10° in temperature brought about an increase of 1.4 fold in the destruction of the vitamin B complex (practically B_1), 58 per cent being destroyed at 130°. A decrease in acidity also increased the rate of destruction (Sherman and Burton),[242] a change from pH 4.28 to pH 5.2 being about equivalent to a 10° rise in temperature. Vitamin B_2 is similarly affected. Guha [98] has reviewed the work of a number of investigators who have found that extracts of the B vitamins from different sources vary in their stability when heated. Sherman and Spohn (see Sherman and Smith)[245] and Elvehjem, Kline, Keenan and Hart [77] found that the presence of moisture affects the rate of destruction of vitamin B_1. Heating dry for 24 or 48 hours at 100° did not materially destroy this vitamin. Elvehjem and co-workers destroyed the vitamin B_2 in a ration by heating it in the dry condition for 144 hours at 95° to 100°, and found it still contained considerable quantities of vitamin B_1.

Halliday, Nunn and Fisher [102, 103] found that they could coagulate the protein in reconstructed skim milk by boiling for 5 minutes at pH 4.3 and recover vitamins B_1 and B_2 practically quantitatively in the filtrate. These vitamins underwent no destruction when this filtrate was kept "in the cold" for a week at pH 4.3 or pH 7; but vitamin B_1 was practically completely destroyed and vitamin B_2 about 75 per cent destroyed when the filtrate was similarly stored for a week at pH 10. Heating it at 97° for one hour at pH 4.3, pH 7 and pH 10 caused losses of vitamin B_1 of 25, 30 and 70 to 80 per cent respectively; the losses of vitamin B_2 were 10,

30 and 40 per cent. Four hours heating of this protein-free filtrate at this temperature and these hydrogen-ion concentrations, caused losses of vitamin B_1 of 30 to 40, 40 and practically 100 per cent respectively; while the losses of vitamin B_2 were 30, 50 and 75 per cent.

Mattick and Golding [184] studied the nutritive value of raw and heat-treated milks. They found that raw whole milk, when fed *ad libitum* along with a white flour biscuit as the complete ration of the rat, supported growth and reproduction.[228] The fourth generation on this diet was as healthy and normal as the previous generations. But when milk that had been sterilized by heating at 210° to 212° F. (99° to 100° C.) for 30 minutes was used in place of the raw milk, the diet failed to sustain life and reproduction beyond the first generation except on one occasion when a second generation of very stunted animals was produced. Even the first generation rats on this diet failed in many instances to reach maturity. These workers also found that milk that was pasteurized by holding at 145° to 149° F. underwent changes which reduced its dietetic value. No evidence is given in this paper as to the nature of the factors destroyed by heating the milk.

Most of the early work on the effect of heat on the vitamin B potency of milk tended to indicate that very little vitamin B_1 was lost in boiling the milk,[162] in autoclaving milk at 125° for 2 hours,[96] in pasteurization,[140] or in the preparation of evaporated [141] or dried milk (spray process).[140] On the other hand, Vedder and Clark [269] found samples of condensed milk poorer in this vitamin than samples of fresh milk that they tested. Most of the earlier experiments were not carried out in a way that would demonstrate small changes in the potency of this vitamin as a result of heating the milk; and frequently the heat-treated samples of milk were obtained from different sources and were not really comparable with the fresh milk with which they were compared.

Dutcher, Francis and Combs [64] compared evaporated milks, made in various ways that approximated commercial manufacturing conditions as closely as possible, with samples of the raw milk from which they were made. These workers found no significant loss of the vitamin B complex in the manufacture of these evaporated milks. Hartwell,[116] and Daniels, Giddings and Jordan,[47] using in both cases the same method and one which has since been severely criticized, obtained evidence that vitamin B_1 is destroyed to some extent in the manufacture of evaporated milk. The work of Donath, in which he used birds as his test animals, would agree with these findings of Daniels and co-workers. He says in summing up his work on heat-treated milks, "In sterilized, in evaporated and in condensed tin milk as well as in milk powder a considerable loss of the antineuritic factor could be demonstrated. In the different brands examined this loss amounted to much more than half the quantity found in the fresh milk." Samuels and Koch [225] have made a very careful study of the vitamins B_1 and B_2 in evaporated milk as compared with a sample of the raw milk from which it was made. They used three methods of determining vitamin B_1, and found that the commercial evaporation

caused a loss of one-sixth to one-fifth of this factor. On the other hand, practically no loss of vitamin B_2 occurred during the process.

In the study of the effect of pasteurization on the B vitamins in milk, Daniels and co-workers found very little destruction of vitamin B_1 when the process was carried out with little exposure to air; but obtained evidence of some destruction when their "open" procedure was used. Krauss, Erb and Washburn [158] and Dutcher, Guerrant and McKelvey [65] have recently studied the effect of pasteurization on these factors. In the former paper, Krauss and co-workers found that milk that was pasteurized by heating at 145° F. (62.5° C.) for thirty minutes was 75 per cent as potent a source of vitamin B_1 as the raw milk from which it was made. The raw milk contained about 0.13 Sherman unit of vitamin B_1 per cc. They found no destruction of vitamin B_2 as a result of this pasteurization. Dutcher and co-workers subjected their milk samples to various heat treatments. One sample was heated at 62° to 63° in a closed flask for thirty minutes; a second one was similarly treated except that when the temperature reached 62° to 63° the flask was evacuated until the milk in it boiled sufficiently to fill it with froth; the third sample was treated like the first except that a stream of air bubbles was drawn through it during the heating; and the fourth sample was boiled under a reflux condenser for ten minutes. With 3 cc. of milk per day as their only source of B vitamins, rats that had previously been depleted made average weekly gains for 6 weeks of 4 grams with the raw milk and 3, 2.7, 3.5 and 3.5 grams respectively in samples 1, 2, 3 and 4. These figures indicate a loss of possibly 12 to 25 per cent in samples 1, 3 and 4 in the potency of the vitamin B complex as a result of the heat treatment to which they were subjected; and a somewhat greater loss with sample 2. They also found a reduction in the concentrations of both vitamins B_1 and B_2 in these heat-treated samples. The results that are given are based on the performance of three rats in each determination (except with the raw milk); but are presented as representative of more extensive work. If we consider only the data that are presented, the differences noted are not large when compared with the error that would exist in their determination. Todhunter [262] found market samples of pasteurized and evaporated milks to have practically the same potency as sources of vitamin B_2.

Donath, as noted above, found that market samples of dried milk are poorer as sources of vitamin B_1 than fresh raw milk. Daniels and co-workers found a greater loss of vitamin B_1 in the manufacture of "spray" process dried milk than in pasteurized milk; but little, if any, loss of this factor in the manufacture of dried milk made by the "roller" process. Hartwell found more vitamin B_1 in dried milk made by the roller process than in spray dried or even evaporated milk.

The above work on the effect of pasteurization and the manufacture of dried and evaporated milks may possibly be summed up by saying that vitamin B_2 in general is probably not materially affected by these processes although in the heating of protein-free milk filtrates it is partially destroyed. On the other hand, vitamin B_1 may undergo a partial destruction

in these processes. In pasteurization this may sometimes amount to as much as 25 per cent; in the manufacture of evaporated milk it may be 15 to 20 per cent or even more as indicated by the work of Donath; while with dried milks the extent of this loss may vary with the method of manufacture.

Vitamin C. For years scurvy was known to occur on diets devoid of fresh fruits, vegetables, milk, etc., and to be prevented or cured by such additions to the diet. Funk [95] attributed this preventive or curative effect of these foods to the presence of an antiscorbutic vitamin in them. This view is now generally accepted, and the vitamin is designated as vitamin C or water-soluble C. Holst and Frölich [124] first studied scurvy experimentally in the guinea-pig. On a diet devoid of vitamin C this animal will grow for about two weeks, then lose weight, and finally die in 4 to 5 weeks. The first symptoms of the disease really precede loss in weight.

The unit of vitamin C is defined by Sherman as the minimum quantity which will fully protect a guinea-pig against scurvy under certain experimental conditions.[245a] The vitamin C activity of milk in comparison with that of a few other representative foods is shown in Table CII. Determinations of the vitamin C content of foods have been rather few, and in many cases the results have been given in such form that it is not easy to calculate from them the C content of the food in terms of Sherman units. Where there has been some difficulty about making such calculations the figures have been bracketed or marked $<$some given figure. In the case of foods marked as having <1 Sherman unit of C activity, it has been impossible to get guinea-pigs to consume enough of them to afford any protection against scurvy, though it does not necessarily follow that such foods might not have some slight antiscorbutic potency for man and other animals. In the case of meat, for instance, guinea-pigs will not consume enough to afford any protection, but human experience has shown that fresh meat has some antiscorbutic activity for man.[187m]

Unless otherwise stated, it is to be assumed that the determinations were made on the foods in the fresh raw state. The authors are greatly indebted to the Bureau of Home Economics of the United States Department of Agriculture for help given in the compilation of this table.

The evidence, although conflicting, leaves little doubt that the supply of vitamin C in the diet of a lactating animal affects the concentration of it in its milk. Barnes and Hume [10] found that summer milk was more effective than winter milk in protecting guinea-pigs and monkeys from scurvy. Hart, Steenbock and Ellis [107] compared the vitamin C content of milk from cows on dry feed (roughages and grains) with that from cows receiving some pasture (timothy, blue grass and clover). The antiscorbutic potency of the latter was about twice that of the former. The feeding of corn silage and of mangels did not greatly affect the vitamin C content of the milk. Hess, Unger and Supplee [118] found very little vitamin C in milk from a cow on dry fodder, and a very quick

increase in the concentration of this vitamin in the milk when the cow was put to pasture. Dutcher, Eckles, Dahl, Mead and Schaefer [68] found milk from pasture-fed cows to be 3 times as potent an antiscorbutic as milk from the same cows on winter rations. Hughes and coworkers [186] have failed to observe this effect of pasture upon the vitamin C content of milk. In view, however, of the positive results of so many other independent workers it may be concluded that a suitable change of diet may alter the vitamin C content of milk. Kennedy and Dutcher [149] found that the effect of pasture feeding upon the vitamin content of milk depends upon the character of the pasture. This they mention particu-

Table CII.—The vitamin C content of representative foods as shown by feeding experiments with guinea-pigs. For explanation, see text.

Food	Description	Vitamin C Sherman Units per 100 grams	Reference
Bananas	....	16	(100)
Carrots	....	6	(153)
Eggs	....	<1	(117)
Lemons	....	53	(218)
Lemon juice	....	[67]	(53)
Lettuce	....	4	(153)
Lettuce	....	7	(218)
Meat	....	<1	(218)
Milk	....	2	(180)
Milk	From cows on dry feed	1	(107)
Milk	From cows on pasture	2	(107)
Peas	Raw	33	(101)
Peas	Cooked	16	(101)
Spinach	Raw	88	(218)
Spinach	Raw	16	(101)
Spinach	Cooked	<10	(101)
Turnips	....	14	(218)
Turnips	....	33	(101)

larly in relation to their study of the effect of it upon the vitamin B content of milk, but it is probably true in the case of vitamin C.

Animals differ in their susceptibility to scurvy. A deficiency in vitamin C in the diet produces it more readily in the guinea-pig than in man or the monkey; the pig and rabbit seem to be less susceptible than either, but not immune to it; whereas a number of birds, prairie dogs, rats and cattle [259a] do not appear to develop scurvy symptoms on diets deficient in vitamin C, although there is some evidence that rats may do better with it than without it.

It has been shown that the livers of rats and chickens contain vitamin C even after they have been for long periods on diets which contain little or none of C. This has suggested that rats and chickens may be able to synthesize vitamin C from compounds which cannot be used for this purpose by such animals as guinea-pigs and monkeys, but this point has never been definitely proved.[187j]

Vitamin C is more readily destroyed by heat and oxidation than any

of the other vitamins. Table CIII taken from La Mer, Campbell and Sherman[161] indicates the percentage of this vitamin destroyed in tomato juice under the conditions of heating indicated and at a pH of 4.18 to 4.37, which is the natural reaction of this fluid.

The increase of destruction with the increase of temperature is less rapid than in most chemical reactions, the temperature coefficient between 60° and 80° being approximately 1.23 and between 80° and 100°, 1.12. The increase in destruction with the time of heating is also less rapid than in any typical chemical relation. This relation appears to be expressed by the equation $K = \frac{x}{\sqrt[4]{t}}$, where x represents the amount of vitamin destroyed.

The rate of thermal destruction of vitamin C increases with the pH of

Table CIII.—Destruction of vitamin C by heat.
(From La Mer, Campbell and Sherman.[161])

No. of experiments	Time of heating	Temperature	Average destruction
	hrs.	°C	per cent
12	1	100	50.2
3	2	100	58.0
11	4	100	67.7
6	1	80	40.5
5	2	80	53.0
5	1	60	25.2
6	2	60	36.8

the solution. Thus in 1 hour's heating at 100° at an initial pH 4.3, 50.2 per cent is destroyed; at pH 5.2, 58.3 per cent; at pH 9.2, 61.8 per cent; and at pH 10.9, 61 to 65 per cent. It decomposes at 10° at pH 8.3, but is more stable at more acid reactions.

This thermal destruction of vitamin C involves an oxidation of it. It is more rapid when its solution is aerated; but occurs under anaerobic conditions, fruit and vegetable juices showing a positive oxidation potential under these conditions. The instability of vitamin C appears to be correlated with this property of these juices.

The vitamin C content of green plant tissues is greatly reduced by such air-drying as is customary in the handling of farm crops. Such forage as fine ground alfalfa or corn stover is so reduced in vitamin C content as to fail to prevent scurvy in guinea-pigs.

The manner in which the C content of milk and milk products is affected by the various procedures to which these commodities are subjected in commerce is discussed at the end of this chapter.

Vitamin C, like vitamin B, is not stored in considerable quantities in the animal body.

It has recently been shown that the antiscorbutic vitamin is a reducing substance having the empirical formula and properties of a hexuronic

acid.[6a] More recently a compound with the structural formula given below has been synthesized.[8]

$$\overset{\qquad\qquad\qquad\qquad\qquad -O\rule{1.5cm}{0.4pt}\urcorner}{CH_2OH.\ CHOH.\ \dot{C}H.\ COH{:}COH.\ CO}$$

The levo form of this compound has the same antiscorbutic activity as the naturally occurring antiscorbutic hexuronic acid (now called ascorbic acid) and may be regarded as the pure form of vitamin C.*

Vitamin D and the physiological effects of irradiation. The same fats (butter and cod-liver oil) which were early found to contain vitamin A are also effective in the prevention and cure of rickets, a condition in growing animals which is characterized by a failure of calcium salts to be deposited normally in the bones and cartilages of the body. This antirachitic action is not due to the vitamin A in these fats, but to a different substance now known as vitamin D, or the antirachitic vitamin. These two vitamins may occur separately in nature. Thus, while spinach and butterfat are rich in vitamin A, the former lacks and the latter is relatively poor in vitamin D. Vitamin D is much more stable toward oxidizing agents than vitamin A. Both of these vitamins have distinct growth-promoting effects, when fed to young animals under suitable conditions; but, whereas a deficiency in vitamin A leads primarily to susceptibility to various infections (xerophthalmia, and infections of the ear, sinus, and lungs), vitamin D is especially concerned with calcium and phosphorus metabolism and conditions leading to the normal calcification of bones in young animals. Both of these vitamins occur in the non-saponifiable fractions of the fats and oils which contain them, and both have similar physical properties.

Exposure of an animal to ultraviolet rays, either from the sun or from a mercury vapor lamp, has the same effect as supplying vitamin D in its diet; and there is evidence that irradiation of this sort leads to the production of this vitamin in the animal body. Vitamin D can also be produced in foods which contain sterols by exposing them to ultraviolet rays having a wave length of from 300 to 310 millimicrons. Preparations of cholesterol, phytosterol, and ergosterol were early rendered antirachitic by this method, and antirachitic fractions were separated from the unchanged portions of these sterols. Such materials as lung, muscle, and liver tissue, cottonseed oils, lettuce and spinach leaves, yeast, bile, lanolin, the grains and their commercial products, milk, egg yolk, and butter, all of which contain sterols, have also been similarly activated. The antirachitic potency of whole milk has been increased fifteen to twenty fold by irradiation. It should be added in this connection that sunlight loses most of its ultraviolet rays in passing through window glass.

It has recently been shown that a very high antirachitic potency can be developed in ergosterol by irradiation, 0.1 gamma of irradiated ergosterol fed daily being sufficient to protect a rat against rickets under certain experimental conditions. Still more recently it has been found

* Personal communication from Dr. E. L. Hirst through Dr. Raymond M. Hann, National Institute of Health, Washington, D. C.

possible to isolate from irradiated ergosterol a pure crystalline substance, which has been called calciferol, and which has about four times the antirachitic potency of the most active irradiated ergosterol. A good account of this subject is given in a recent publication of the British Medical Research Council.[187d]

There is reason to think, however, that calciferol is not the same substance as that which gives to cod liver oil its antirachitic potency. Cod liver oil cures rickets in rats and also leg-weakness in chickens, which is regarded by many investigators as a condition closely related to mammalian rickets. Irradiated ergosterol, on the other hand, while it cures rickets in rats, is ineffective against leg weakness in chickens, except in very large doses.[20] Further, Hess and others have shown[121] that the antirachitic potencies of irradiated ergosterol, irradiated milk, and cod liver oil for rats do not run parallel to their antirachitic potencies for infants.

Another set of observations pointing in the same direction is that concerning the antirachitic potencies of human milk and of cow's milk. General experience has shown that infants are less likely to develop rickets on human milk than on cow's milk. But Outhouse, Macy, and Brekke find that cow's milk has a much higher antirachitic potency for rats than human milk, and that this is the case even when the high phosphorus and calcium content of cow's milk is compensated for in the experimental procedure.[209]

The above is only one of many aspects of the situation which complicate the study of the antirachitic factor in nutrition. The favorable effects of vitamin D on calcium and phosphorus assimilation are usually demonstrated on young rats whose diet is rather high in calcium and low in phosphorus. Sherman and MacLeod have found, however, that cod liver oil has very little favorable effect on the calcium deposition in the bones of rats which are fed on rations somewhat low in calcium.[240]

Further, it has been shown in numerous experiments that cows which are giving large quantities of milk are likely to be losing calcium and phosphorus from their bones.[188] Under these circumstances, however, neither cod liver oil[188] nor irradiated yeast[118] has any favorable influence on calcium and phosphorus assimilation, while the assimilation of these elements is facilitated by an organic dietary factor which is present in larger quantity in hay of good quality than in hay of poor quality.[109, 188, 265]

All of these results suggest that the assimilation of calcium and phosphorus by animals is influenced not only by the quantities of calcium and phosphorus in the diet and by irradiation with ultraviolet light, but also by several organic chemical compounds which have different effects on different species of animals and on the same species at different ages and under different physiological conditions. In view of these complications, it is not surprising that there is some confusion in the scientific literature in regard to the requirements of different animals for the antirachitic

vitamin and in regard to the measurement of the antirachitic potencies of different food substances.

The Health Organization of the League of Nations has recently proposed a standard unit to be used in vitamin D determinations. This unit is defined as 1 milligram of a standard solution of irradiated ergosterol in pure olive oil prepared by the British National Institute for Medical Research. One milligram of the solution contains 0.0001 milligram of irradiated ergosterol, and approximately one-fourth of this dose given for 8 successive days to a rachitic rat under certain experimental conditions will produce a narrow continuous line of calcium deposits in the metaphysis of the proximal ends of the tibiae and of the distal ends of the radii.[187o] Detailed directions as to how vitamin D determinations are to be carried out will be given in the forthcoming edition of the U. S. Pharmacopeia, of which advance sheets are already available.[267]

These advance sheets also give the relationship between the different units for the measurement of vitamin D which is reproduced below:

One international unit of vitamin D equals approximately:
0.37 Steenbock unit.
3.25 American Drug Manufacturing Association units.
1.66 Oslo units.
One Holtz unit equals about 6.7 international units, and one Holtz clinical unit equals about 670 international units.[20a]
Average American cod liver oil contains about 100 international units of vitamin D per gram.

The situation in regard to the quantities of vitamin D contained in milk and other foods is usually summed up at present with the statement that milk, butter, and eggs are the only common foods which contain any measurable quantities of vitamin D, and that even they contain very little in comparison with cod liver oil. In considering this subject, it is necessary to distinguish between the vitamin D content of foods and their total antirachitic potency. The antirachitic potency of foods is generally measured by means of rats which are given a basal ration rather high in calcium and very low in phosphorus. This feature of the basal ration is introduced intentionally in order that it may cause the rats to become rachitic rapidly. Foods added to this basal ration may have antirachitic potency not only on account of their vitamin D but also on account of their phosphorus and calcium content, and each of the experiments must be studied with this consideration in mind before its results can be interpreted and correlated with those of others.

Crawford and collaborators find that the D content of milk is contained exclusively in the fat.[42] This conclusion has been generally accepted, and investigators often calculate the D content of milk from that of the fat and the percentage of the fat contained in the milk. As the question of the D content of milk fat is not complicated by any phosphorus or calcium contained in this material, the figures which have been obtained for the D content of milk fat will be considered before those which have been obtained for whole milk. D contents will be given

throughout in terms of international units per gram of food as used, and only such results will be considered as can be fairly easily converted into terms of international units by means of the data given above.

The D content of butter varies greatly according to how the milking animal is fed and according to how much ultra-violet irradiation she receives. Under ordinary conditions the butter from animals on pasture is likely to be richer in D than that from animals kept in barns under winter feeding conditions. The D content of butter can, however, be driven up much higher than it ever is under ordinary conditions by feeding to the milking animal such D concentrates as cod liver oil, irradiated yeast or irradiated ergosterol.

As the limits of variation for butter produced under ordinary conditions, the British Medical Research Council gives 0.1 to 1.5 units per gram.[187e] A number of British and American investigators have obtained results which lie within these limits.[89, 157, 159, 276] Cows which are fed on D concentrates may produce butter which contains from 2 to 8 D units per gram.[157, 159]

Calculated for milk, the above figures would mean that 4 per cent milk from cows under ordinary conditions would contain from 0.004 to 0.06 D units per gram, and that milk from cows which had received D concentrates would contain from 0.08 to 0.3 D units per gram. Some of the results given for milk lie within these limits,[119, 125, 198] but the British Medical Research Council makes a statement which indicates that the antirachitic potency of milk produced under ordinary conditions may be as high as 0.2 unit per gram,[187e] and Hess gives results which indicate that the antirachitic potency of milk from cows fed D concentrates may rise to above 0.4 unit per gram.[120] It seems possible that these figures represent the total antirachitic potency of the milk rather than its vitamin D content.

The situation with regard to the vitamin D content of eggs seems to be closely parallel to that of milk. Bethke finds that the yolk of eggs from hens on blue grass range may contain 10 times as much D as that of hens confined indoors on the same basal mash.[19] A recent Wisconsin bulletin gives results from which it may be calculated that egg yolk from hens under winter conditions contains about 0.2 international D unit per gram; that from hens under summer conditions 0.9 unit; and that from hens given irradiated yeast 5.4 units.[288]

In connection with the subject of vitamin D in foods, it should be mentioned that many fish oils are rich sources of this vitamin.[68b]

The standard average American cod liver oil contains 100 international units of vitamin D per gram. One teaspoonful of this, therefore, would contain about as much vitamin D (though not as much antirachitic potency) as 6 liters of cow's milk produced under the most favorable ordinary conditions of feeding and treatment. It would contain as much vitamin D as a liter of cow's milk in which the D content had been driven to the highest point so far obtained by feeding D concentrates to the cow.

Much evidence has accumulated which shows that in England, at any

rate, rickets is a very common condition among children.[104] Defective teeth are also common in England as well as in this country, and there is a good deal of evidence to show that a lack of antirachitic potency in the diet of children may be a cause of defective teeth not only in childhood but in later life.[187g] For this reason much effort has been made to increase the vitamin D content of human diets, and rather extreme measures have been recommended by many authorities.

It is unfortunately true, however, that calciferol, irradiated ergosterol, and cod liver oil all have toxic properties when given in large doses. The British Medical Research Council has given reviews of the evidence for the toxic properties of cod liver oil[187b] and of irradiated ergosterol and calciferol.[187c] Harris discusses the toxic properties of irradiated ergosterol for human beings. He states that in human beings the margin between the curative dose and the toxic dose is less wide than it is in the case of rats. He recommends 3,000 international units of vitamin D daily as the curative dose and states that a daily dose of 10,000 international units is on the border line for hyper-vitaminosis.[104]

In considering this situation from the practical point of view, it must be remembered that it is impossible to carry out experiments to determine satisfactorily the toxic properties of any drug or food material for human beings. Toxicity must be judged from cases where it has happened that bad effects have followed the giving of unusually large doses and have been reported. Bad effects which might result from more moderate doses given over long periods would be much less likely to be attributed to their true origin. It seems obvious, therefore, that in making general recommendations for dealing with the D requirements of human beings, a middle course should be taken which will guard just as carefully against the dangers of over dosage as against those of deficiency.

Milk, butter, and eggs are the only ordinary foods which contain considerable quantities of vitamin D, and even in these the D content is so moderate that an infant living entirely on milk could not receive more than 100 international units of vitamin D daily, unless the female which supplied the milk had been fed on one of the modern D concentrates. An adult living entirely on ordinary milk and eggs could not receive more than about 600 units of D daily. Most human beings have gone through their lives without any very marked suffering from rickets on quantities of D much smaller than this.

The evidence for the wide prevalence of rickets among children in northern countries cannot, of course, be neglected, but should not be accepted entirely uncritically. It is normal in human beings for much of the calcification of the bones to take place after birth, and there are no doubt natural and non-pathological variations in the rapidity with which this process occurs in different individuals. The use of X-rays has made it possible to detect slight retardations in this process, which would not have been noticed 50 years ago and which are probably not pathological.[187a] It does not seem wise to hurry every baby into the fastest possible calcification by the use of medicines which supply 5 or 10 times as much

vitamin D as the human race has been accustomed to in the past, and which are known to be toxic in large quantities. A teaspoonful of cod liver oil given daily to a baby will increase the quantity of D which it would receive with its ordinary diet more than four-fold, and it would seem that approximately this amount through the winter ought to be sufficient as a prophylactic dose for the ordinary healthy infant. Cases where rickets has actually developed, of course, should be put under the care of physicians, and the discussion of them lies outside the province of this book.

Vitamin E. Rats may grow well and have every appearance of health on synthetic food mixtures consisting of purified fats, carbohydrates and proteins along with appropriate salt mixtures and adequate amounts of vitamins A, B, and D, but sooner or later animals on such diets fail completely to reproduce. Evans and Bishop[84, 85] observed such failures with rats on a diet of casein 18, corn starch 54, lard 15, butterfat 9, and salt 4, along with 0.4 gram of dried yeast daily. The females were

Table CIV.—Classification of certain foods as to their effect on sterility.

Materials which prevented or cured the sterility in female rats.		Materials which failed to prevent or cure the sterility in female rats.	
Lettuce	Rolled oats	Milk	Lactalbumin and in-
Meat	Alfalfa (dried)	Orange juice	creased amounts
Whole wheat	Wheat germ	Cod-liver oil	of protein
Large amounts (20%) butterfat		Excess of yeast	Cystine

sterile, chiefly so in the first generation but wholly so in the second. Estrus, ovulation, fertilization and implantation occurred normally; but 12 to 20 days after implantation the fetus died and was reabsorbed. Male rats also, when put on such a diet at weaning, were usually sterile at the normal age of sexual maturity or soon became so.

Evans found that this sterility could be prevented or cured in the case of female rats by certain changes in their diets, as indicated in Table CIV.

Two hundred and fifty mgs. of wheat germ, as well as a single drop of wheat germ oil (25 mgs.) given daily cured it. The active material occurs in the unsaponifiable fraction of the oil, and is readily soluble in methyl and ethyl alcohols, ether, pentane, benzene, acetone, ethyl acetate, carbon disulfide, etc. Evans called this potent fat-soluble material vitamin X, but it has since acquired the name of vitamin E. While in female rats the sterility due to a deficiency of vitamin E is apparently merely functional and is readily cured, as well as prevented, by the addition of this vitamin to the diet, in the male it involves a destruction of the germ cells and finally a degeneration of the entire seminal epithelium which when complete can not be restored.

Vitamin E is present to a small extent in a great variety of animal tissues (muscle, fat, pancreas, spleen, liver, heart, hypophysis, placenta); but is especially low in its concentration in the viscera, being lower, for instance, in the liver than in muscle. It is stable toward heat, light, and

air. Heating wheat germ to 170°, distilling wheat germ oil with superheated steam at 180°, or distillation of fractions containing vitamin E in vacuum at 233° do not appear to affect it; and it is remarkably stable toward acids and alkalies, although some destruction occurs in a very prolonged hot saponification. It is not destroyed when wheat germ oil is hydrogenated at 75° in the presence of palladium. Its chemical nature is unknown. Preparations have been made that were so potent that a single dose of 5 mgs. when injected into a rat at the beginning of gestation led to the birth of a normal litter of vigorous young. It contained only a trace of ash and no N, S, P, or halogen.

The work on vitamin E up to 1927 is very fully given in a monograph of that date.[86]

It was shown some time ago that the quantity of vitamin E required by an animal was influenced by the amount of fat in the diet. Thus in diets in which butterfat furnished the vitamin E, Evans and co-workers failed to obtain reproduction using a mixture of casein 18, salts 4, butterfat 9, lard 15, starch 54, with 0.4 gram of yeast fed separately daily; while Nelson and co-workers [199] were successful with a diet of casein 18, salts 3.7, butterfat 5, yeast 2, and dextrin 71.3; and unsuccessful when 10 per cent of lard was substituted for dextrin. Nelson's work would indicate that 5 per cent of butterfat furnishes an adequate supply of vitamin E when no other fat is present in the diet; whereas Evans secured reproduction when 15 per cent of butterfat replaced the 15 per cent of lard, giving 24 per cent of butterfat in his feed mixture, and upon a whole milk powder diet containing 28 per cent of butterfat. He concludes that this fat is very deficient in vitamin E.

More recent work has shown that vitamin E is affected by pro-oxidants and anti-oxidants in the diet, which are associated with fats and perhaps also with other nutritional factors.[5c] Fats like cottonseed oil or butter, which take up oxygen relatively slowly, have no antagonistic action toward vitamin E; while fats like cod liver oil and lard, which take up oxygen more quickly, are antagonistic.* It has been shown also that male rats fed on milk to which traces of iron and copper have been added become completely sterile.

The question of the quantity of vitamin E in milk or butter is also complicated by considerations other than those already discussed. Reproduction may be influenced by dietary factors other than vitamin E. It may fail, as has been shown with rats, as a result of general undernutrition or of deficiencies in the protein, mineral supply or in vitamins A or B. Daniels and Hutton,[46] in their work referred to above, have tried the effect of the addition of certain salt supplements to fresh whole milk. With small supplements of iron, iodine, aluminum, fluorine, manganese, silicon and traces of such other inorganic constituents (e.g., copper) as may have occurred in their salt preparations, they have secured with rats a good average number of normal young through six genera-

* The statement given in the Annual Review of Biochemistry on this subject does not agree with that in the original article of Cummings and Mattill.

tions. This interesting work indicates that the vitamin E content of milk is adequate where this food constitutes a large proportion of a diet not too high in fat. It is not possible at the present time to say much more than this about the E content of milk.

Although vitamin E does not occur in great concentration in the animal tissues tested for it, it may be stored in amounts that are physiologically very considerable and be retained for long periods of time. This is shown when rats are shifted from a diet adequate in vitamin E to one inadequate in it. They continue to reproduce for some time. After sterile animals are cured by the administration of vitamin E, the fertility may survive for two succeeding gestations without further administration of the vitamin. A single large dose of it at the beginning of pregnancy is as effective as daily small doses totaling the same amount. There is, however, in time some wastage of vitamin E in the animal economy.

Milk as a source of nutritive energy. The energy value of milk per unit of weight is rather low on account of its large water content. Sherman[246a] discusses the "physiological fuel value" of milk and other foods, and finds that average cow's milk, after allowing for the losses in digestion, will yield to the body about 69 calories per hundred grams. This would amount to about 690 calories per liter or 313 calories per pound. It would take, therefore, three and three-quarters liters of average milk to supply 2500 calories which are required daily by the average man with a sedentary occupation.

The digestibility of the nutritive material of milk is very high. The extent to which rats digest the constituents of cow's milk has recently been investigated by Nevens and Shaw, who find that, in the case of fresh whole milk, the protein is 91.8 per cent digestible; the fat, 99 per cent; and the lactose, completely digestible. Earlier work is quoted which shows that human beings digest about the same percentages of the constituents of cow's milk as do rats.[200] It is frequently claimed that the digestibility of milk is greatly increased by certain manufacturing processes, but, in view of the situation above outlined, such claims should be accepted with caution.

The protein of milk supplies approximately 19 per cent of its total calories. While the protein content is high enough to permit optimal growth in young animals, it is not so high as to cause large losses of energy through the high specific dynamic action of protein.

Comparative value of milk proteins and other proteins in maintenance. Much of the experimental work that has been done on the nutritive value of proteins has involved the use of milk proteins. They have, therefore, been investigated more thoroughly than any other food proteins; but the methods used, results obtained, and conclusions drawn have been variable in the extreme. Mitchell[191] has published a very excellent critical review on the nutritive value of proteins. (Reference to the work of most of the investigators mentioned in this section on proteins will be found in Mitchell's article. Where this is not the case the references will be found at the end of this chapter.) Older and more general

work on the physiology of protein metabolism is reviewed in Cathcart's monograph by this title.[35]

The amount of food protein that is digested and absorbed is generally obtained by subtracting the fecal N from the food N and by multiplying by 6.25. This, expressed in per cent of the total food protein, is the "coefficient of digestibility" given generally in text books. Calculated thus, about 90 to 100 per cent of animal food proteins (including milk proteins), 80 to 90 per cent of cereal, vegetable and fruit proteins, and 80 per cent or less of legume proteins are absorbed in human nutrition. These figures may vary considerably, not so much with the kind of protein, as with the amount of indigestible non-protein material in the diet. In the case of some of the legume proteins the low digestibility is improved by cooking and appears to be associated with some peculiarity in their chemical make-up.

The above method for calculating the "digestibility" of a food protein does not take into account the fact that not all of the fecal N is unabsorbed food N, and that, even on a protein-free diet, a considerable amount of N is thus excreted. No entirely satisfactory way of estimating this "metabolic" fecal N has been devised. Mitchell claims that it is approximately proportional to the total amount of food ingested. He determines it on a protein-free diet containing varying amounts of this material, and calculates it accordingly on other rations. With this correction he finds with rats fed 5 per cent of milk proteins in their rations that 95 per cent is absorbed and with 10 per cent in their ration 92 per cent is absorbed; without this correction the average digestibility was 70 per cent in each case. One may make similar calculations with the human subject using data from very carefully executed experiments by Martin and Robison. Whereas the average digestibilities of whole meat and of milk proteins in their experiments were 78 per cent and 80 per cent respectively when no account was taken of the metabolic fecal N, they were 93 per cent and 99 per cent when this was corrected for. Rose and MacLeod [220] have published experiments with the human subject in which the digestibility of milk proteins was 89 per cent, of meat proteins 88 per cent, of bread and milk proteins 88 per cent, and of soy-bean proteins 76 per cent when no correction is made for metabolic fecal N. These figures, and the corresponding figures of Martin and Robison, are relatively lower than usual because the average N intake in both cases is unusually low, making the metabolic fecal N proportionately larger.

Data occur in the literature which have been interpreted to indicate that the digestibility of a mixture of food proteins is not the same as the weighted average of the digestibilities of the proteins in the component feed materials. This, in most instances, is probably due to variations in the character or amount of indigestible non-protein material in the ration. It is common practice to calculate the digestibility of a feed mixture from the coefficients of digestibility of its components. This is in general warranted for practical purposes where the feeding materials are similar in their non-protein constituents (e.g., crude fiber content). Where this

is not true the calculated digestibility may be somewhat in error. Mitchell has called attention to a case of this sort in an experiment of Hart and Steenbock with pigs. The dietary proteins were derived from corn and milk. In Table CV the latter is assumed to be completely digested, and the absorption of the corn protein calculated accordingly is seen to vary from 51.6 per cent to 77 per cent as the proportion of milk protein in the ration was increased.

Table CV.—Digestibility of corn protein as affected by per cent of milk protein in diet.

Milk protein in diet in percent of total	Total protein in ration in per cent	Percent of corn protein absorbed
3.2	8.6	51.6
6.4	8.9	48.0
11.7	9.4	49.5
20.7	10.2	58.1
28.5	10.9	77.0

The nutritive value of a protein is measured by the maximum proportion of its absorbed digestion products which, under the most favorable conditions, is used to meet the specific N requirements of maintenance, or in the building up of protein in bodily repair and growth or in reproduction and lactation. It depends mainly upon the chemical make-up of the mixture of amino acids which the protein yields in digestion; and varies with the function or combination of functions which these serve.

Experiments have been carried out to determine the nutritive value of milk proteins in human maintenance. In most of them the amount of milk N necessary to meet the minimum endogenous N losses from the body has been measured. The results have varied considerably; but, in general, indicate that milk and meat proteins are very efficiently used for this purpose, that they are more efficiently used than vegetable proteins, and that the mixture of proteins in milk is more efficiently used than casein alone.

Thomas first published data of this sort. In Table CVI are given his figures for the nutritive or "biological" value of various food proteins as measured thus, expressed as the percentage quantity of body protein which their ingestion will spare from loss when they are added to a protein-free diet.

Table CVI.—Comparative nutritive value of various food proteins.

Source	Value	Source	Value
Ox meat	104	Cherry juice	79
Cow's milk	100	Yeast	71
Fish	95	Casein	70
Rice	88	Spinach	64
Cauliflower	84	Peas	56
Crab meat	79	Wheat flour	40
Potatoes	79	Corn meal	30

According to these data a gram of fish, meat or milk protein may reduce the loss of body protein by practically one gram when added to a protein-free diet; whereas 2½ to 3 times as much of wheat or corn protein would be required for the same purpose. Thomas' experiments were carried out upon himself; the work is the most extensive of its kind with a human subject; his results have been given wide publicity and have done much to formulate the general view held regarding the nutritive value of these proteins. But a casual review of his experimental practice and methods of handling his data would convince anyone that his figures can not be taken quantitatively. For instance with milk protein the experiment lasted but two days, for which the energy and protein intakes and N balances were not the same. The data for one day were used in his calculation; and the figure for the urinary endogenous N loss was about twice that determined since in more careful experiments, and was determined a fortnight before, when his N stores were less depleted. His data may possibly be taken as indicating in a rough way the relative value of these proteins when used in meeting the maintenance requirements of the organism.

Martin and Robison determined in duplicate upon themselves the nutritive or "biological" value of milk and wheat proteins following the same general experimental procedure as Thomas'. Their experiments were much more carefully executed. Their duplicate results with wheat proteins agreed well; the average, expressed in a way comparable to that of Thomas, was 33. This agrees well with Thomas' figure for the cereal proteins. Martin and Robison found that 8 to 9 grams of wheat protein N were necessary to bring about N equilibrium. Hindhede [122] has found it possible to do this on less than half this amount, and accordingly gives a much higher value to these proteins when used to meet the essential N requirements for maintenance. A number of other investigators have obtained data indicating a better utilization of these proteins in maintenance than would be indicated by Thomas' and by Martin and Robison's data. N equilibrium can be established at various levels over a wide range of protein feeding; and Hindhede claims that Martin and Robison did not wait long enough to come into equilibrium when their diets contained the smaller amounts of protein, but too quickly increased their protein intake to a level where equilibrium was more readily attained.

Whereas Thomas stored protein when he ingested 6 to 7 grams of milk N, Martin and Robison found it necessary to take 7 grams to attain N equilibrium even with an excessive intake of energy in the diet. Their endogenous N losses were very little more than one-half those of Thomas, so that in one case for a period of 28 days they obtained a biological value of only 51 instead of 100 as found by Thomas. Martin and Robison's work needs repetition. Early in their experiments, on approximately 3 grams of milk N with an energy intake which they considered inadequate, there was a loss of less than 1 gram of body N per day. From these data one would obtain a nutritive value about 75 per cent or more for milk proteins.

The work of Rose and MacLeod [220] would also sustain a higher value for the use of milk proteins in maintenance than that obtained by Martin and Robison. With 4.15 grams of N in the daily diet, 97.6 per cent of which was from milk, Rose and co-workers obtained an average N retention of 0.55 grams daily during a period of 12 days. This certainly would give a nutritive value of not less than 90 if expressed in a manner similar to the results of Thomas and of Martin and Robison. Rose and MacLeod have confirmed these results in additional experiments on milk proteins, and have obtained similar results with approximately the same amounts of meat and of bread and milk proteins. There are a number of observations which when brought together tend to indicate that food proteins may be as efficiently utilized in maintenance as the tissue proteins of the body, and it would appear that this may be true of milk proteins. Despite the results obtained by Hindhede which would lead to a similar view relative to wheat and rye proteins, a number of observations tend to indicate that milk proteins are more efficient for this purpose than these cereal proteins.

Table CVII.—Per cent of proteins from various sources necessary in a diet capable of maintaining body weight.

	per cent		per cent
Milk proteins........	3	Oat proteins..........	4
Rice "	6	Millet "	4+
Cottonseed "	6	Flaxseed "	8
Maize "	6	Pea "	11
Wheat "	6	Bean "	11
Alfalfa "	6+		

In experiments in which the food intake was not carefully controlled, McCollum and co-workers, working with rats, were able to maintain them without loss of weight when the percentage of protein in their rations was that indicated in Table CVII.

Following a much more carefully controlled procedure Osborne and Mendel have determined the maintenance values of proteins by following the body weight of their subjects. They fed ample but constant amounts of energy to rats. The concentration and the quality of the protein in the ration were the only factors varied. With each kind of protein the amount was so adjusted that the animal maintained constant weight. They found that it took 9.3 mgs. of lactalbumin, 15.5 mgs. of casein or 14.6 mgs. of edestin, on an average, per gram of rat per week for this purpose. Evidence also occurs in their work which indicates that the proteins of milk as a whole are intermediate in their value between casein and lactalbumin, and 2½ times as efficient as gliadin. They obtained figures indicating a somewhat lower value for whole wheat (23 to 26 mgs. per gram of rat per week) than for milk proteins, but conclude that their data "show that for maintenance the proteins of the wheat kernel are not greatly inferior to those of casein—or even to the total proteins of milk." Wheat endosperm proteins were somewhat less efficient than the whole wheat proteins.

Mitchell and his co-workers have determined the nutritive, or "biological" value of proteins for the maintenance of rats by feeding various proteins as 5 per cent of the diet. This would allow very little, if any, growth. Table CVIII expresses the percentage utilization of these proteins in a manner comparable to that used above by Thomas and by Martin and Robison.

The surprising feature about these data is the small differences between the utilization of the various kinds of proteins in maintenance. In a number of his experiments with milk proteins fed at a 5 per cent level, Mitchell obtained a "biological" value of 100 and his data consistently indicate a value for milk proteins in maintenance relatively higher than

Table CVIII.—Relative biological values of proteins from various sources.

Kind of protein	Biological value
	per cent
Milk	93.4
Rice	86.1
Yeast	85.5
Oat	78.6
Soy-bean	78.0
Coconut	75.0
Corn	72.0
Casein	70.8
Potato	68.5

that of any of the vegetable proteins. Mitchell's data differ from Thomas' mainly in assigning higher values to the cereal proteins than the latter obtained in his experiments. That the efficiency of the cereal proteins for maintenance is relatively higher than at first supposed is now generally believed. Other than this, the agreements between the results of Thomas and Mitchell are much more striking than the differences, although the former was rough pioneer work with the human subject, and the latter is recent with rats, and is the result of very careful critical consideration of work in this field.

Comparative value of milk proteins and other proteins in growth. It is impossible to measure the value of a given protein for growth alone, as maintenance in addition to growth is always involved. When an animal is maintained at higher levels of protein feeding there are increased losses of amino acids, yielding energy but serving no other particular function in the organism. Such losses also occur when proteins are fed in sufficient quantity to permit growth. The extent of such losses with any particular food protein depends upon a number of factors. The optimum utilization under the most favorable conditions is characteristic of the kind of protein and depends upon its chemical make-up. Mitchell and co-workers, in the same work with rats referred to above in which they fed proteins at practically a maintenance level, also determined their value when fed as 10 per cent of the ration, which level of feeding would

permit considerable growth. The data in Table CIX [191] indicate the percentage of food protein utilized in maintenance and growth together.

Here again the data indicate that milk proteins are more efficient than the vegetable proteins; and, as the differences here are fully as great as when these proteins were fed at a 5 per cent level, it is evident that the differences in their utilization for growth are fully as great as for maintenance, if not greater.

Table CIX.—Percentage of various food protein utilized in maintenance and growth.

Kind of protein	per cent
Milk	84.7
Rice bran	67.0
Potato	66.7
Cottonseed meal	66.0
Oat	64.9
Soy-bean	64.0
Alfalfa	62.0
Corn	59.0
Corn meal	49.0
Coconut	58.0

McCollum in 1914 carried out some very carefully conducted experiments on the utilization of various proteins by growing pigs. He expressed the protein retained in per cent of that absorbed. His results are given in Table CX.

Table CX.—Relative degree of utilization of various proteins.

Kind of protein	Percent retained
Oat	20.75
Corn	26.41
Wheat	22.13
Mixture of corn, wheat, and oat	32.56
Wheat embryo and wheat gluten	23.19
Casein	50.6
Lactalbumin	66.19

In these figures the utilization of some of the dietary protein for maintenance is not allowed for. With certain assumptions, Mitchell has calculated the percentage retention of the absorbed protein which he estimates to have been used in maintenance. His figures are given in Table CXI.

Table CXI.—Relative retention of absorbed proteins.

Kind of protein	Protein retained, as per cent of that available for growth
Corn	48
Wheat	44
Oats	42
Casein	67
Milk	80

These figures, however, would vary depending upon the percentage efficiency for maintenance that is used in the calculation.

McCollum and Davis [176] found that 3 per cent of milk proteins is sufficient for maintenance with rats and that with increases in the concentration of milk protein in the ration from 3 to 8 per cent there were corresponding, progressive increases in the rates of growth, 6 per cent inducing one-half normal growth. About the same result was obtained with 6 per cent whole wheat as with 4 per cent of milk proteins, or 4 per cent wheat embryo proteins. These results show clearly the superiority of milk proteins over whole wheat proteins for the combined functions of growth and maintenance. In 1919, McCollum and co-workers in summing up their previous work state that the best proteins for these functions in rats are those of eggs and milk (he had not tried meat proteins). The cereal proteins they considered only one-third to one-half, and pea and bean proteins only one-fourth as efficient as milk proteins. In the experiments which they reported at this time they compared the growth of rats on diets containing wheat, rye, corn, flaxseed oil meal, barley, kaffir corn, oat and milk proteins. Each was fed at a 9 per cent level with the exception of the milk proteins. These made up only 8 per cent of the ration. The rate of growth and weight at maturity, however, were greater upon milk proteins than upon any single vegetable protein that they tried.

In 1921 McCollum and co-workers published an extensive series of papers on the nutritive value of various proteins when fed separately and combined in various ways. They varied their feeding materials so that each protein made up 9 per cent of the ration. The rest of the diet was such that the supply of protein alone limited the growth. In the first paper they compared the growth of rats on proteins from ox-kidney, liver and muscle. The first produced the most rapid growth and greatest maximum weight. Muscle proteins came next, and liver proteins somewhat lower. The results with kidney protein exceeded somewhat those previously obtained with milk proteins; whereas those with muscle and liver proteins fell below this. In a later paper of this series they compare the growth obtained with wheat, oat, barley, pea, and soy-bean proteins, but in no instance did the mature weight or rate of growth exceed that previously reported for milk proteins. They obtained somewhat better results with whole wheat proteins in these experiments and in their 1919 experiments than with the other cereals. In the experiments reported in 1921 they did not determine again the growth effect obtained as a result of feeding milk proteins. It is clear, therefore, that with milk proteins McCollum and co-workers consistently obtained better growth and a more efficient utilization than with any of the cereal or legume proteins, fully as good as with muscle or liver proteins, and that only kidney protein gave better results.

In addition to following the growth effect of these proteins in their later experiments, McCollum and co-workers noted the effect of their diets upon fertility, success in rearing young, span of life up to and including

the onset of old age, etc. Taking into consideration all of these factors and attributing their variations to the variations in the kind of protein in the rations used, they arranged these foods accordingly, in the order of the "biological value" of their proteins as follows: The best is placed at the left and the series is a descending one.

Beef Kidney	——Wheat——	Milk Liver	——	Muscle (round steak) Barley Rye	——	Maize Oats	——	Soy-beans Navy beans Peas

Since this arrangement in some respects does not accord with the values of these proteins for growth and maintenance, it is evident that the authors have placed a preponderant significance upon the other factors considered. Since these papers were published it has been shown that dietary factors then unknown are involved in reproduction. There may be factors that are still unknown that influence lactation and the rearing of young. Nothing really is known regarding the dietary constituents that may influence the span of life, the onset of sterility, etc. The proteins that McCollum and his co-workers used were fed as they occur ordinarily in our feeding materials along with innumerable other substances, the effect of which is unknown. To attribute all of the variations in the performance of their animals to the changes in dietary protein and thus neglect the possible effect of these other dietary changes is unwarranted. Other complications also beset an expression of a general "biological value" of a protein in the broad sense in which McCollum and his co-workers used this term. Thomas, Martin and Robison, and Mitchell use the phrase "biological value" in a much more restricted sense to apply to the utilization of proteins in maintenance and growth.

Osborne and Mendel have determined the utilization in growth of the individual milk proteins (i.e., casein and lactalbumin, which included all the proteins coagulable by heat after removal of the casein). They have made similar determinations with some other proteins, so that comparisons are possible. They have used three different procedures, in all of which they employed rats. In one, starting with animals weighing 40 to 50 grams, they ran the experiment for 11 weeks. The food intake was carefully restricted and controlled so that it was the same with rats of the same size and was increased in precisely equivalent amounts in the successive periods of the experiment in accord with the anticipated needs due to increasing body weight. The only variables, as between one rat and another, were the kind and concentration of proteins in the ration. The growths observed are given in Table CXII.

Apparently 9.9 per cent of lactalbumin, 19.8 per cent of casein, or 20.5 per cent of edestin produced the same result; but, when cystine was added to the casein, the mixture was as efficient as lactalbumin. This method gives comparable figures but does not permit maximum utilization of the protein because of the restriction of the diet.

In the second method used by Osborne and Mendel, they employed rats of the same age, sex and weight (80 grams), fed them the same amounts of foods, varying the kind and concentration of the protein so

Table CXII.—Relative utilization of certain proteins in growth.

Kind of protein	Per cent in ration	Gain in weight in gms. per 11 weeks
Lactalbumin	14.8	122
Lactalbumin	9.9	100
Casein	19.8	100
Casein	16.2	105
Edestin	20.5	101
Edestin	16.7	95
Casein	8.0	71
Casein (plus 3 per cent of cystine)	8.0	95

that equal gains in weight were made in equal periods of time. Eight per cent of lactalbumin, 12 per cent of casein, and 15 per cent of edestin were found to produce the same result. When cystine was added to the casein, 18 per cent less of this protein produced a 12½ per cent greater gain in weight than without the cystine.

In the third method Osborne and Mendel varied the concentration of each kind of protein in the ration so as to obtain the maximum gain in weight and the gain in weight per gram of protein fed. About 20 per cent of protein occurs in muscle, so that if the growth consists entirely of an increase in muscle tissue, the gain in weight is 5 times the protein laid on. Without deducting the protein used in maintenance, Osborne and Mendel found a maximum of 3 grams gain in weight per gram of lactalbumin in the ration, whereas the maximum gain in weight per gram of casein was 2.25. The same amount of food was eaten regardless of the concentration of the protein in it, but 16.2 per cent of lactalbumin produced almost twice the gain in weight produced by 6.2 per cent. Mitchell has calculated from these data of Osborne and Mendel that about 90 per cent of lactalbumin, fed over and above that required for maintenance, may be converted into body protein.

Some experimenters (McCollum and others, and Sure) have reported results which would indicate that lactalbumin is an "incomplete" protein. They failed to obtain any growth with young rats on diets containing 18 per cent of it, and otherwise adequate. Sure has attributed this failure to a deficiency of cystine in lactalbumin; and he, and McCollum and his co-workers have explained Osborne and Mendel's success as due to the use of protein-free milk by the latter in their synthetic diets. The latter experimenters [207] have repeated their work on lactalbumin, omitting the protein-free milk, and have obtained excellent growth with 9 per cent of it in the diet. This work is such that it leaves no doubt regarding the high value and completeness of this protein for growth.

There is no doubt that the cystine content of casein is relatively sufficiently low so that it limits the utilization of this protein in growth; and the work of Sherman and Merrill [239] indicates that this is also true for the proteins of milk as a whole, although they obtained two-thirds normal growth on as little as 5 per cent of milk proteins in the diet, which would indicate that the mixture of milk proteins is, nevertheless, very

efficiently utilized in growth and maintenance. There is much reason to believe that lactalbumin has a high cystine content; the work of Jones and his co-workers [142] and of others indicates this, and the experimental data taken as a whole suggest that lactalbumin supplements the cystine deficiency in casein when these proteins are used together in whole milk.

With barley, rye, oat and wheat proteins, Osborne and Mendel obtained considerable growth when these were fed as 5 per cent of a ration which was low in fat and would be consumed in considerable quantity as compared with high-fat rations. Where the energy intake was high in these experiments, 10 per cent of these proteins in the ration permitted normal or nearly normal growth. The average gain in weight per gram of protein fed in these experiments was as follows: For barley protein, 1.55; for oat protein, 1.39; for rye protein, 1.33; and for wheat protein, 1.11. As comparable figures for lactalbumin and casein, Osborne and Mendel give 2.3 and 1.7 respectively, whereas a figure as low as 0.5 was obtained with the proteins of the wheat endosperm which occur in white flour.

The work of Osborne and Mendel, as well as that of a number of other investigators, while showing that the cereal proteins are adequate for growth and maintenance, leaves no doubt of the superiority of lactalbumin and the relatively high value of casein for these purposes. On the whole it may be taken that 75 to 100 per cent of the absorbed milk protein may be utilized under suitable conditions in growth and maintenance, 50 to 65 per cent of cereal proteins, and 30 to 40 per cent of the proteins in the legumes. The milk proteins taken together have about the same biological value as meat protein, but casein by itself has a somewhat lower value, on account of its small cystine content. Cystine seems to be the first limiting factor for growth in the milk proteins taken together as well as in casein.

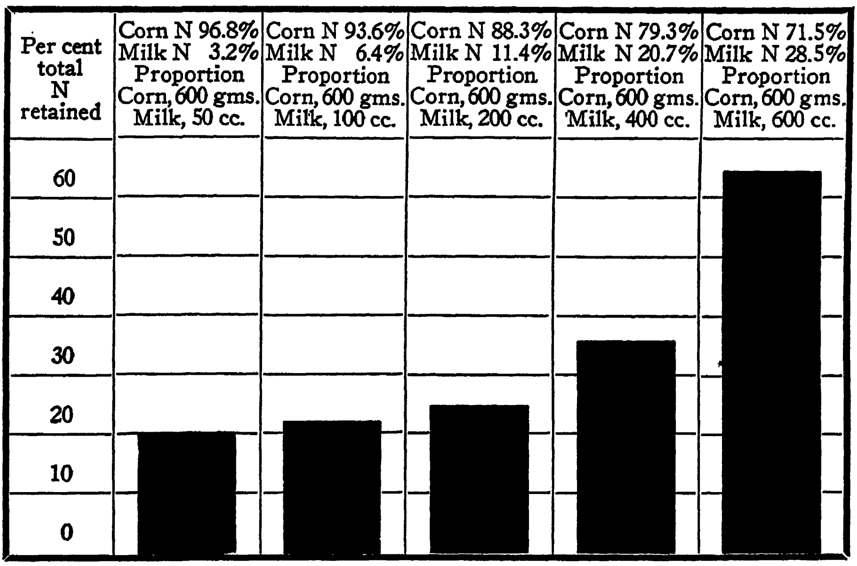
Shiftan [250] reviewed the work that had been done to determine the relative value of different proteins for maintenance and for growth. Using swine and following approximately the procedures of Thomas and of Mitchell, he determined the biological values for casein and for milk, fishmeal, potato, and barley proteins when fed as 2 per cent of the ration. The results, respectively, were 63.3, 100.6, 66.2, 69.6 and 58.7 per cent. When these proteins were fed as 12 per cent of the ration (i.e., for both maintenance and growth) the corresponding figures were 81.8, 95.1, 73.8, 73.2 and 70.8 per cent respectively.

Chick and Roscoe, Fixsen, and Fixsen and Jackson suggested changes in the methods of determining the biological value of proteins. Using adult male rats and five-day feeding periods, they made a number of determinations of the biological value of casein. In one paper they give 45 per cent as the average of 12 determinations in which the casein was fed at between 4.4 and 15.6 per cent of the ration; and no correlation was apparent between the level of feeding and the value obtained. In another paper they give a value of 65 per cent for casein when fed as 6 per cent of the ration. In this paper they also give a value of 86 per cent for lactalbumin and 70 per cent for milk proteins as a whole when con-

stituting 7 per cent of the diet. Wheat germ, wheat endosperm, and maize endosperm, fed as 7 per cent of the ration gave values of 69, 61 and 70 per cent respectively. Whole wheat at 6 per cent of the ration gave a value of 68 per cent, and whole maize fed at 8 and 5 per cent of the ration gave biological values of 67 and 84 per cent respectively. The references are given in the paper by Fixsen and Jackson.[90]

Supplementary relations of proteins. The manner in which the biological value of casein is limited by its cystine content serves as a good example of the manner in which the nutritive properties of proteins depend on their amino acid make-up. It is obvious that casein might be advantageously supplemented in the diet by some other protein having a

Table CXIII.—Retention of dietary protein as affected by proportions of protein fed.



high cystine content as well as by cystine itself. Mitchell has published quantitative experimental data showing a supplementary relation of this sort between the total proteins of corn and of milk when fed in the ratio of three to one respectively and as a total of 10 per cent of the ration. The biological value of milk proteins when thus fed alone is 84.7, of corn proteins 61.3 and the weighted mean for the above mixture 67.1. Mitchell found experimentally a biological value of 75.7 for the mixture. The difference between the calculated mean, 67.1, and the determined biological value, 75.7, measures the supplementing effect as a result of feeding the milk protein and corn protein together. These values, of course, would vary with the ratio between the amounts of the component proteins in the mixture and with the level at which the mixture is fed in the ration.

Table CXIII shows the per cent of dietary protein retained when

various proportions of corn and milk proteins were fed to pigs. It is copied from Hart and Steenbock.[106] They conclude that a highly efficient protein mixture is not obtained until the proportion of liquid milk to corn meal reaches 1:1. In this proportion the milk N constitutes 30 per cent of the total N of the diet. Osborne and Mendel likewise obtained normal growth when milk proteins furnished 25 per cent and corn proteins 75 per cent of the dietary N. Although excellent growth is thus obtained on such a mixture, a number of investigators have reported failure to obtain optimum or normal growth on corn proteins alone. Other cereal, and especially legume proteins, are likewise inefficient for growth.

This fact, and the importance of animal proteins in nutrition as supplements to the cereal proteins is brought out by the data of Table CXIV in the case of wheat flour proteins. The table is taken from Osborne and Mendel.[206] Wheat gluten is added to the wheat flour to make the quan-

Table CXIV.—Relative nutritive efficiencies of various proteins.

Sources of food protein	Per cent of protein in food	Gain of body weight per gram of protein
Flour plus egg	14.8	2.00
	10.3	1.80
Flour plus milk	14.8	1.67
	10.3	1.73
Flour plus meat	14.8	1.73
	10.3	1.47
Flour plus meat plus yeast	10.3	1.66
Flour plus gluten	14.8	0.50

tities of protein fed comparable. The animal proteins constituted one-third of the protein mixture. The gain in weight per gram of protein mixture fed is taken as the measure of its efficiency.

Measured thus, the wheat flour proteins are from one-fourth to one-third as efficient as their mixture with the animal proteins in the ratio of 2:1. Apparently there is little difference in the relative values of these animal proteins as supplements for the proteins of wheat flour, although egg proteins here appear to be somewhat the most effective.

In the extensive work by McCollum, Simmonds and Parsons on the supplementary relation of food proteins it is concluded that there is no marked supplementary relation between the proteins from different cereals; that the legume proteins are of low nutritive value; that there is no supplementary relation between the proteins from different legumes (peas, navy beans, soy-beans); that there is supplementary relation in some instances between cereal and legume proteins (e.g. proteins from wheat and navy beans, proteins from wheat and peas); but that proteins of kidney, liver, muscle meat or milk are remarkably effective as supplements to the proteins from the cereals and legumes, the combinations with the cereals being especially efficient. Milk they held to be less effective

than the animal tissue proteins in this respect although at the same time it supplements other deficiencies in these seeds (e.g. calcium and vitamin A).

Animal foods, therefore, not only are valuable because they furnish rich sources of utilizable protein that quantitatively supplement this deficiency in vegetable foods; but also because they supplement qualitatively the deficiencies that occur chemically in the amino acid make-up of vegetable proteins so that these are used more efficiently in meeting the N requirements of the animal organism.

Mineral content of milk in connection with its nutritive properties. The bulk of the animal body is made up of the organic proteins, fats, and carbohydrates, which are composed of the elements carbon, hydrogen, oxygen, nitrogen, and sulfur. But it has long been known that a number of other chemical elements are constantly found in the body in various amounts. These are often spoken of as the "mineral elements" of the body, though not with entire exactness. Some of them take part in the formation of the mineral salts found in the body; others occur only as parts of complicated organic compounds; and still others occur partly in organic and partly in inorganic combination. As a rule the elements which occur in largest amounts in the body have been recognized first and most carefully studied, while those occurring in smaller amounts have received diminishing degrees of attention. We know today that there are a great many chemical elements which are constantly or usually found in the body in small quantities whenever the chemical methods used are sufficiently refined. We know also that several of these are of great physiological importance; while, in regard to others, it is still uncertain whether they play any important physiological part or not. All of the essential chemical elements must, of course, be supplied to the growing animal in its food, and it is a question of great interest how far these essential elements are contained in milk, and how far the quantities and particular combinations of them which occur in milk are adequate to supply the needs of the growing animal body.

The importance of the so-called inorganic chemical elements in nutrition has been quite fully discussed by Sherman [246] and the elementary composition of the human body is given in an earlier part of this book. A comparison of the composition of the body and of milk shows that most of the chemical elements found in the body are found also in milk. Bunge [31] has pointed out that the ash of dog's milk contains many elements in about the same proportion as the ash of the young dog's body.

Among the mineral elements, calcium and phosphorus take a somewhat exceptional position, on account of being present in large quantities in the body as constituents of the bones. The question of the amounts of them required in the food of the growing animal has been a subject of experimental investigation for a long time, and there is reason to believe that deficiencies are not uncommon in the diets of human beings as well as of the lower animals. Milk contains liberal amounts of both calcium and phosphorus. In the case of calcium particularly, milk contains more per calorie or per unit of dry matter than the great majority of other

articles of human food. As far as can be judged from chemical considerations, therefore, milk should be a good source of calcium and phosphorus in the diet.

The assimilability of the calcium of milk has been compared with that of other foods by McClugage and Mendel and by Sherman and Hawley. McClugage and Mendel [174] used adult dogs as the subjects of their experiments, and compared the calcium assimilation in diets in which this element was chiefly supplied in the form of milk, calcium carbonate, carrots, and spinach respectively. They found that calcium was better assimilated from the milk diets than from any of the others—somewhat better than from the diets containing calcium carbonate, and much better than from those containing carrots or spinach.

Sherman and Hawley [285] have carried out an extensive investigation of the calcium and phosphorus metabolism of children on different diets. They worked first with different quantities of milk from 500 to 1500 cc. daily per child, and found that the calcium assimilation did not reach its maximum until the child got as much as 1000 cc. of milk daily. They then tried various methods of comparing the assimilation of calcium and phosphorus from milk with that from spinach and carrots included in the diet. The basal diets contained bread, butter, oatmeal, potatoes, sugar, and orange juice. In one series of experiments, the calcium and phosphorus balances were determined first on the basal diet plus 500 cc. milk; then on the same diet, but with enough carrots and spinach substituted for the potatoes to supply as much calcium as would have been contained in 500 cc. of milk; and, finally, on the same diet as in the first period. It was found that, in spite of the much higher calcium content of the diet in the second period, the children assimilated no more of this element than in the first and third periods. In another series of experiments, the carrots and spinach were substituted for a certain amount of milk roughly equivalent in its calcium content. In this case the calcium assimilation was reduced in the carrot and spinach period to what it presumably would have been had these vegetables been omitted from the diet. In both these series of experiments, the phosphorus balances showed some tendency to follow the calcium balances.

The results of the whole investigation indicate that the calcium and phosphorus of milk are very well assimilated by children, while in the case of carrots and spinach they are not assimilated at all—at least, where these vegetables are added to a milk diet which contains enough calcium and phosphorus to cover a considerable fraction of the optimal assimilation.

The assimilation of calcium and phosphorus from milk and from vegetables by adult human beings has been studied by Rose and by Blatherwick and Long. In one series of experiments, Rose [219] determined the calcium balances in two adults who were first for a period of 21 days on a diet in which the calcium came largely from milk, and then for the same period on a diet in which the calcium came largely from carrots. In one subject about equal positive calcium balances were obtained on both diets; while in the other the balance was positive on the milk diet and

slightly negative on the carrot diet. All of the balances were small, as would be expected in the case of adults. The largest one for the 21-day period was +0.087 gram calcium daily. In a second series of experiments Rose showed that small positive calcium balances could be obtained in adults when the calcium intake was supplied chiefly by carrots and amounted to only 0.3 gram per day or less—rather less than the average amount given by Sherman as necessary to maintain equilibrium.

Blatherwick and Long [23] determined the calcium and phosphorus balances of adults, first on basal rations containing quite small amounts of milk, and then on the same rations with considerable quantities of such vegetables as spinach, lettuce, and asparagus added to them. The addition of vegetables, of course, increased the calcium and phosphorus content of the rations; and it was found that the calcium and phosphorus balances were negative on the basal rations and became positive when the vegetables were added.

The experiments of Blatherwick and Long can hardly be regarded as a comparison between the assimilability of calcium from milk and from vegetables, and neither they nor those of Rose furnish any evidence to contradict the conclusion of Sherman and Hawley to the effect that calcium from milk is more readily assimilated by human beings than that from vegetables. They do indicate that the small calcium requirement of adult human beings is fairly easily covered by the calcium that can be obtained from vegetables, and they hint perhaps that the calcium of vegetables is better assimilated by adults than by children. But it is worth while to point out that experiments on adults are less satisfactory as an index of the assimilability of calcium from different foods than experiments on children. In the latter case there is a considerable daily demand for calcium to build the growing bones, and conditions are favorable for determining whether any given food contains sufficient assimilable calcium to meet this demand. In the case of adults, on the other hand, the normal condition is equilibrium; and whether the body is likely to gain a little calcium or to lose a little calcium at any particular period is very apt to be determined by conditions other than the food given during that period.

Sherman and his collaborators have made an elaborate study [240, 241] of the calcium and phosphorus assimilation of rats on the two diets which he calls Diet A and Diet B, and which have already been described. Diet A is composed of one-sixth whole milk powder and five-sixths whole ground wheat with sodium chloride equal to 2 per cent of the weight of the wheat; while Diet B contains one-third whole milk powder and two-thirds whole ground wheat with sodium chloride equal to 2 per cent of the weight of the wheat.

They find that rats on Diet B contain larger percentages of both calcium and phosphorus in their bodies at all ages than rats on Diet A. Adding vitamins A and D to Diet A in the form of cod-liver oil causes a somewhat better growth in the male rats and a very slightly better growth in the females, but does not cause any increase in the percentages of calcium and phosphorus contained in the bodies of either males or

females. Adding calcium lactate to Diet A causes a smaller increase in the rate of growth of the males than does cod-liver oil, but a slightly larger increase in the case of the females. It brings the percentages of calcium and phosphorus in the bodies of both males and females up to those which are obtained on Diet B.

These results confirm and extend those obtained by Sherman in his experiments on children by indicating that calcium and phosphorus are present in milk in highly assimilable form, that fairly large amounts of milk must be fed in order to insure optimum calcium and phosphorus assimilation in growing animals, and that fairly similar statements can be made on this subject for widely different species of mammals.

The fact that milk is deficient in the mineral elements which are necessary for the building of hemoglobin, namely iron and copper, has already been discussed. Since the discovery that animals do not become anemic on diets of milk with small amounts of iron and copper added, there has been some further work on the mineral elements which are essential in nutrition, and evidence has been brought forward which indicates that milk is deficient in several other mineral essentials in addition to iron and copper.

Orent and McCollum fed rats on a manganese-free ration composed of casein, butterfat, corn starch, alcoholic yeast extract, viosterol, and a salt mixture free from manganese. On this ration the males became sterile through testicular degeneration. The females gave birth to living young, but would not suckle them. It was shown that the lack of manganese played a part in bringing about these pathological conditions.[202]

A few years later Keil and others showed that rats fed on milk plus small amounts of iron and copper gave birth to young, but that the second generation did not reproduce. The addition of small amounts of manganese sulphate to the ration made reproduction possible for the second generation. When a salt mixture containing small amounts of copper (see Hart and co-workers [112]) and of iron, iodine, aluminum, fluorine, manganese, and silicon (see Daniels and Hutton [46]) was added to the milk, reproduction continued through four generations, but the fifth generation was sterile.[148]

Of the seven elements above enumerated iron, copper, and manganese have already been sufficiently discussed. Fluorine is a constant constituent of bones and teeth, and is probably essential in nutrition, but is toxic when consumed in more than very small amounts.[246b] In regard to aluminum and silicon, very little is known. Iodine, on the other hand, is an important nutritive essential, and, in many parts of the world, its deficiency in the soil and in foods is the cause of goiter in human beings [246c] and in farm animals.[105] Work reviewed by Maynard [186] indicates that considerable changes in the iodine content of milk can be brought about by changes in the iodine content of the food of the milking animal. As goiter is common among children and young farm animals in regions where iodine is deficient, but not in regions where it is plentiful, it seems not unlikely that milk may contain sufficient or insufficient iodine for the needs of the

young according as this element is plentiful or scarce in the food of the milking animal.

General Considerations in Regard to the Use of Milk as a Food, Particularly for Infants and Young Children

It should be clear from the foregoing discussion that milk is in general more nearly complete in all dietary essentials than any other single food. A rather surprising feature of the situation, however, is that the vitamins, which play so large a part in endowing milk with its unique dietary properties, appear in it in highly variable concentrations, according as they are plentiful or scarce in the food of the milking animal. There has accumulated a considerable body of evidence to suggest that the human race might be able to improve its general health and well-being by paying more attention to the quantity of the various vitamins in its food supply, and particularly in that of its children; and this evidence has already led to various procedures aimed at increasing the supply of some of the vitamins in our food. A discussion of this situation has a place in the discussion of the nutritional value of milk.

Deficiencies of vitamins in milk are, of course, most likely to become apparent in infants and young children whose food consists chiefly of milk. There is as yet no evidence to show that children suffer from vitamin deficiencies when they are nursed up to the age of 8 months or more by adequately fed mothers, and then given diets which contain large proportions of fresh milk from adequately fed cows, and when both mothers and children receive plenty of sunshine out of doors. But there is plenty of evidence that vitamin deficiencies may occur where the mothers are inadequately nourished, where the cow's milk has been altered in some way for one reason or another, or where the exposure to out-door sunshine has been insufficient. Among the deficiencies which have been observed, those which have attracted most attention in Europe and America are deficiencies in A, C, and D.

Symptoms typical of A deficiency have been observed in Denmark in children fed on milk from which the fat had been removed.[187k] The chief of these was xerophthalmia, but it is interesting to note that there was a high incidence also of respiratory infections.[187l] Deficiency of vitamin A, therefore, gives rise in children, just as it does in the lower animals, to xerophthalmia and increased susceptibility to infections, particularly of the respiratory tract; and the vitamin A content of milk requires attention in cases where this food is the main article of diet. In view of the fact that the A content of cow's milk is likely to be much reduced during the winter under ordinary conditions of feeding, it seems wise to take some precautions either to prevent this condition or to add vitamin A from some outside source to the diet of children during the winter. Unpublished experiments carried out by the Bureau of Dairy Industry have shown that the A content of the milk of cows on winter feeds can be much increased

by adding moderate quantities of yellow carrots to the ration; and the addition of cod liver oil to children's diets is already extensively practiced.

In regard to vitamin C, there is clear evidence that human infants are likely to develop scurvy when they are fed on heated cow's milk as the sole article of diet.[187n] There is very general agreement that this condition can be prevented by the feeding of small amounts of orange juice, and this procedure is so easy to carry out and so generally satisfactory that it is widely recommended and practiced as a part of the general routine in the feeding of infants. Orange juice has about the same antiscorbutic potency as lemon juice, that is about 30 times as much as fresh milk from cows on pasture. Other antiscorbutics have been used with satisfactory results in the rather unusual situations where orange juice is not available.

In America, in Europe, and particularly in England, rickets, imperfectly developed teeth, and carious teeth are very common conditions among children. It has been shown that all of these conditions can be prevented or ameliorated by increasing the supply of vitamin D in the diet or by subjecting the patients to sufficient irradiation with ultraviolet light.[187f] Various forms of such treatment have been recommended and practiced, of which the following are important examples: 1. The administration of cod liver oil to children, particularly through the winter. 2. The feeding of sources of vitamin D, particularly irradiated yeast, to dairy cows. 3. The irradiation of milk with ultraviolet light. 4. The irradiation of the children with ultraviolet light from an artificial source. 5. The exposure of the children to direct sunshine by letting them spend a considerable part of their time outdoors.

There are a good many reasons to recommend the first of these procedures through the coldest and darkest months of the year in northern climates. At that time of year sunshine is scarce and feeble, and foods are likely to be deficient in vitamins A and D, both of which are supplied by cod liver oil. Further, this remedy has been used for a long time by the human race, and may be regarded as well tried out; it is being used at the present time with many children as a matter of routine, apparently with satisfactory results.

The second procedure is much more questionable. The effects of concentrated preparations of D on the health of dairy cows have not yet been sufficiently studied to justify their use as a matter of routine from the point of view of the dairyman, and to administer curative materials to human beings by putting them first through cows is certainly a very wasteful therapeutic method.

The third procedure is decidedly more questionable than the second. Milk is the food about which most of our modern knowledge of nutrition has grown up. Again and again rations which were "theoretically" complete have been found deficient in practice; again and again milk has turned out to contain the missing unknown factors; and there is every reason to think that we do not yet know all of the important dietary essentials which are contained in milk. Ultraviolet rays are known to damage the important A[251] and B_2 [187h] vitamins; and, while a degree of irradiation

might be found which would supply sufficient antirachitic vitamin without seriously damaging the other two, it is impossible to guard against damage to the factors which are not yet known. The irradiation of milk is all the less justifiable in view of the fact that the administration of cod liver oil is so easy and economical; indeed, it is difficult to see any respect in which this first procedure is not superior to either the third or the second.

The irradiation of the human body with ultraviolet light from an artificial source is troublesome, expensive, and somewhat dangerous. It seems unlikely that this procedure will ever be used as a matter of routine or under any except unusual circumstances.

The fifth procedure, that is, securing more outdoor life and sunshine, especially in the middle of the day in early spring when the sun is already fairly high, and human stores of vitamin D are likely to be depleted, is, for obvious reasons, not advertised as much as it ought to be. It is true that "sun bathing" in summer has become rather a fad; but the same individuals who expose most of their bodies to the sun in summer, when ultraviolet light is over-plentiful, will often stay all day in houses and offices, and not take the trouble to expose even their faces and hands to sunshine in late winter and early spring when their stores of antirachitic vitamin are likely to be depleted.

There are already on the market a number of concentrated vitamin preparations designed for human consumption, and it is altogether likely that these will become more numerous, less expensive, and of better quality as times goes on. It seems likely that the discriminate use of such preparations under certain circumstances will turn out to be highly beneficial, but their probable limitations and disadvantages deserve a word of comment.

It is unlikely in general that the vitamins as they exist in concentrated preparations are themselves exactly the same chemically or occur in the same chemical associations as in natural foods. All of these bodies are complicated compounds with infinite possibilities of small modification, and it has often turned out that such small chemical modifications are very important physiologically. Further, we do not yet know all of the dietary factors which are essential for a complete ration, and are far from knowing the optimum quantities of any of them. It has been shown already that large doses of irradiated ergosterol,[187c, 62] of colorless A,[51] and even of cod liver oil [187b] are toxic. It is true that the doses which have been shown to be toxic are much larger than those which are needed in nutrition, but it must be remembered that in human nutrition it is desirable to avoid not only death and such severe symptoms as come on quickly and can be conveniently studied experimentally, but also minor ills which may take a long time to appear and are difficult or impossible to study in experiments with animals.

It seems likely, therefore, that for a long time, if not always, it will be better for the human race to secure its supply of vitamins by a proper selection of such natural foods as are recognized by their taste and by long experience to be wholesome and of good quality. Knowledge of the

vitamin content of different foods is already very helpful in devising rations, and the use of concentrated preparations will no doubt be beneficial under certain circumstances, where for economic or other reasons it is impossible to secure all of the best of the natural foods. But the view, sometimes expressed, that we shall soon be substituting the products of chemical manufacture for milk and other natural foods, and irradiated ergosterol for fresh air and sunshine, seems quite unwarranted.

Effect of Manufacturing Processes on the Nutritive Value of Milk and Milk Products

Most of the milk consumed in this country is altered in one way or another before it reaches the consumer. A large proportion of the whole milk used is pasteurized, and another large portion of the milk produced is used for the manufacture of such products as butter, cheese, and condensed milk.

The milk products sold on the market have less water and therefore more nutritive energy in proportion to their weight than milk itself. But, in the manufacturing processes, large quantities of nutritive material are often added to or taken away from the solid constituents of milk, so that the chemical constitution of the product is changed in many ways. And even where this is not the case, the nutritive properties of the very complicated and easily altered constituents of milk are likely to be changed.

The composition of the milk products has been discussed in some detail in Chapter I. This part of the subject will be given here only in outline sufficient to make clear the connection between the composition of these products and their nutritive properties.

Pasteurized milk. Pasteurization should not perhaps be included under the head of manufacturing processes in a strict classification; but, as it affects a large proportion of all whole milk consumed in this country, and may have some influence on its nutritional properties, it must be considered here.

A great deal of work has been done on the effects of heat on the chemical and nutritive properties of milk. Rupp,[223] for instance, finds that pasteurization at a temperature somewhat above that usually employed has no significant effect on the solubility of the calcium and phosphorus compounds of milk, does not coagulate the lactalbumin, and causes the casein to coagulate only slightly more rapidly with rennin.

Later Bell[18] has worked on the solubility of the calcium and phosphorus compounds of milk, and has confirmed Rupp's results. Still more recently Magee and Harvey[182] have published an article on the same subject, and have discussed some of the previous literature. There is a very satisfactory agreement that pasteurization, as ordinarily practiced, has only a small effect on such physical and chemical properties of milk as have so far been studied.

A large amount of work has been done on the nutritive properties of raw and heated milk with the general aim of solving the practical

question whether it is advantageous to heat milk which is intended for the nourishment of human infants. In the course of this work a number of species of animals have been involved and some observations have been made on babies. In many of the experiments milk was not the sole article of food used; the points observed were the gain in weight, general health, and mortality of the animals studied. A good review of the work up to 1916 is that of Lane-Claypon.[168] Her conclusion is that little if any impairment of nutritive value is produced by heating milk to the boiling point or below. Many of the experiments studied seem even to show a slight improvement in nutritive quality as the result of heating.

Since 1916, attention has been largely focused on the vitamins contained in milk, and many investigators have worked on the question of the effects of heat on these substances. This subject has been treated in some detail in the preceding sections on the various vitamins, and need only be summarized here.

There is good reason to believe that such heat treatment as is used in pasteurization has no deleterious effects on vitamins A, B_2, D, and E. It does, however, partially destroy vitamins B_1, and C. It is generally recognized that milk is not a rich source of vitamin C. Even fresh milk sometimes does not contain enough of this vitamin to prevent scurvy when it is used as the sole food for human beings; and pasteurized milk is, of course, still more frequently inadequate. In regard to vitamin B_1, the destruction which takes place on pasteurization is not likely to be more than 25 per cent of that originally present.

In considering the practical conclusions to be drawn from this situation, it must be remembered that it is not usually wise to use milk as the sole article of diet, even for young animals. The specialists on infant feeding recommend that babies have a small amount of orange juice as a part of their diet from the first few days of life onward, even when they are nursed by their mothers, and the same recommendation applies with still more force to babies who do not receive their mother's milk. It is easy to feed orange juice in such quantities that a diet of pasteurized milk with this supplement contains more vitamin C than fresh milk without it.

In regard to vitamin B_1, the destruction on pasteurization is not likely to be more than 25 per cent; cow's milk contains considerably more than human milk; and orange juice is, fortunately, a fairly rich source of this vitamin (see Table C). Pasteurized cow's milk can be so supplemented with orange juice that the diet will contain more of vitamins B_1 and C than fresh human milk, and, therefore, presumably sufficient for the needs of the human infant.

Most of the experiments where raw and heated milk have been fed along with other articles of diet have failed to show any superiority of the former, and general practical experience seems to be in harmony with this outcome of the experiments. There can be little question, therefore, that the dangers which are known to be avoided by pasteurization should receive more consideration than the rather questionable points of superiority of raw milk. Until infectious diseases are under much more com-

plete control than at present, it will be wise for our larger communities to hold to and perhaps even extend the practice of pasteurization.

Cream and skim milk. One of the oldest and best-known treatments to which milk has been subjected is that of the separation of the cream. The process is so familiar that it needs no systematic treatment, but it can be made the occasion for certain statements which apply very generally to the products of milk manufacture.

Almost all of these consist of certain constituents of the milk which have been separated from certain others, sometimes with and sometimes without additional food substances. They are usually easier to keep, cheaper to transport, and more convenient and suitable to use with certain other articles of food than milk itself; many of them, also, are prized for their flavor or for the taste of the other materials which have been added to them. But milk itself is the best balanced and most nearly complete of all our foods, and very few of its products are so suitable to form a preponderant or even large proportion of the diet. The unbalanced nature of many of the milk products and their deficiencies in various nutritive essentials are obvious from an account of their manufacture and composition, and systematic comment on this side of the subject will be unnecessary.

Practically all cream sold on the market in this country is separated from the milk by centrifugalization. Such cream contains from 17 to 54 per cent fat, with an average of 37 per cent, while gravity cream contains from 14 to 24 per cent with an average of about 20 per cent.[164] The water of cream contains about the same proportions of solids-not-fat as does that of whole milk.

Skim milk consists of water and solids-not-fat in about the same proportion as that in which they occur in whole milk. It contains in undiminished proportions all of the nutritive essentials of whole milk, except fat and the fat soluble vitamins. Fat, of course, is practically absent, and the greater portions of vitamins A, D and E are also removed with the cream. Practical experience is in agreement with experimental evidence [82] in showing that skim milk is a good source of assimilable calcium and phosphorus for farm animals. This product has, however, generally been looked down upon as a food for human beings—an attitude which is justified when one considers the serious effects which result when its deficiency of fat soluble vitamins is not corrected. Sherman and MacLeod [238] have shown that rats do very well on a ration of ground wheat and whole milk powder; but that, when skim milk powder is substituted for the whole milk powder, they tend to die of infections of the respiratory tract and fail to reproduce normally. The advantages of skim milk as a large part of the ration of many farm animals are well understood. As already noted, it is an excellent food for human beings, provided that the fat soluble vitamins are supplied from other sources.

By far the larger part of the market cream at present used is pasteurized, the main exception being whipping cream or cream for some other

special purpose. Skim milk is not sold on the market on account of the popular prejudice against it.

Butter. Butter making practices vary to some extent, but in general there are only two types made,—sweet cream butter and sour cream butter. As the names indicate, the first type is made from fresh sweet cream, and the second, from ripened or soured cream. The souring is accomplished by the addition of a starter made with lactic acid bacteria. The larger part of the butter sold is made from pasteurized cream. The process of buttermaking consists of the separation of the fat from the milk by means of agitation. It is usually carried out at from 7.2° to 15.6° C. (45° to 60° F.) so that heat plays no part in changing the nutritive properties.

Butter is the most limited of the dairy products, in so far as the variety of its nutritive essentials is concerned. It contains, on the average, 83 per cent fat, 13 per cent water, 1 per cent protein, and 3 per cent salt. The salt consists almost entirely of sodium chloride added during the process of manufacture and only to a very slight degree of the salts present in the original milk. The protein and lactose, and most of the important mineral elements contained in milk are, therefore, for practical purposes, not represented in butter.

Butter contains no appreciable quantities of the vitamins B and C. It does, however, contain small amounts of vitamins D [210] and E,[85] and it is the most important source of vitamin A in the average American dietary.

A pound of butter supplies 3,410 calories, enough to support a man at moderately hard labor for 24 hours. Hunziker [139] gives the coefficient of digestibility of butterfat as 97.86 per cent.

Cheese. Cheese is a class name for a great variety of milk products. The different kinds of cheese have been discussed in Chapter I and Chapter VIII, and only an outline of the subject need be given here.

Cheese making consists essentially in separating most of the protein and fat of milk from most of the lactose and water. The separation is not so complete as in the case of butter; most cheese contains considerable quantities of water and some lactose, while appreciable amounts of proteins and fat are lost in the whey. The minerals and vitamins of the milk divide themselves according to their solubilities. The larger proportions of the calcium and phosphorus of the milk remain in the cheese, while most of the potassium and sodium is lost in the whey. The water soluble vitamins B and C, also are probably largely lost, while the larger proportions of the fat soluble A, D, and E remain in the cheese.

Cheese is very commonly made from whole milk, in which case its protein and fat bear about the same relations to each other as in milk. It may, however, be made from cream, from skim milk, or from any combination of the two, with corresponding variations in its fat content.

The precipitation of the protein and fat may be brought about by means of rennet or by the lactic acid which is produced in milk by bacteria. The former is by far the more common method of precipitation. In either case, the main milk protein which goes into the cheese is casein; the lactalbumin is lost in the whey.

Cheese is very commonly heated at some stage of its manufacture, but the heating is almost always moderate, and the changes in nutritive properties which it brings about are less important than in the case of the pasteurization of milk. Considerable quantities of sodium chloride are often added, and the mineral content as given in the ordinary analyses must not be taken to represent minerals left over from the original milk.

From the foregoing outline of the subject of cheese manufacture, it will be seen that it is, like butter, a product in which the nutritive energy of the milk has been very much concentrated. A much greater variety of the nutritive essentials of milk is retained in cheese than in butter; but some of them are lost, and it is, therefore, less suitable to form a large part of the diet of growing and of reproducing animals than is milk itself.

The nutritive energy of cheese varies greatly according to its fat and water content. American Cheddar, which may be taken as an example of a whole milk cheese with the average water content, contains about 1995 calories per pound, while cottage cheese, made from skim milk and with a large amount of water, contains only 500 calories.[282] Doane[54] carried out extensive experiments in feeding different kinds of cheese to human subjects. He found that the digestibility of the nitrogen ranged from 82.6 to 96.6 per cent; the digestibility of fat from 80.9 to 93.7 per cent; and the availability of energy from 75.0 to 91.0 per cent. The subjects had no ill effects from eating large quantities of cheese.

Further information regarding the vitamin content of cheese is to be found in Sherman's book.[245]

Ice cream. Ice cream differs from the milk products so far considered in that it has undergone not only the removal of certain milk constituents but also the addition of large quantities of other food materials not derived from milk at all. The most common ingredients are cream, sugar, condensed milk, gelatin, and some flavoring substance. Eggs, cornstarch, and flour are also used in some varieties. The amounts of the different constituents used vary with the kind of ice cream and with the manufacturer.

The fat of ice cream varies from 8 to 14 per cent; and the carbohydrate (chiefly, of course, added cane sugar), is usually about 20 per cent.[88]

Like most of the other milk products so far considered, ice cream is a concentrated source of nutritive energy; but the large amounts of fat and sugar contained in it would make it impossible for a growing or reproducing animal to consume enough to supply its needs for protein, minerals, or vitamins, with the exception perhaps of vitamin A. There is no reason to think that any of the processes used in the manufacture of ice cream would destroy the vitamin A contained in butterfat.

Sweetened condensed milk. This product is made by adding about 15 per cent of cane sugar to milk, and then removing a large part of the water by evaporation. In commercial practice the milk is usually "forewarmed" immediately after the addition of the sugar to a temperature somewhat below the boiling point. From the forewarmer the milk is run

into the vacuum pan, where it is condensed to about two-fifths of its original volume, so that the finished product has about 28 per cent total milk solids and about 9 per cent fat. The condensation is usually carried out at about 56° and 25 inches of vacuum, but this varies in the industry, a higher temperature being used where the vacuum is less, and vice versa.

The composition of condensed milk is sufficiently indicated by the foregoing account of its manufacture, and practically the same comment applies to its nutritive properties as to those of ice cream. Compared with whole milk, condensed milk, concentrated as above, is about twice as potent per unit of volume as a source of vitamins A and B_2, and at least 60 per cent more potent as a source of B_1. It is reported to contain a considerable portion of the vitamin C of the original milk.[246d]

Evaporated milk. This product is forewarmed at a temperature of about 95° for approximately 10 minutes. It is then run into the vacuum pan and heated to from 54.4° to 60°, depending on the degree of vacuum obtained, until reduced to one-half of its original volume. It contains from 25 to 34 per cent total solids and about 8 per cent fat. After being evaporated, it is sealed in cans and sterilized at a temperature near 116° for 15 minutes. There are rather wide variations in the times and temperatures used in the different operations, however, on account of the differences of opinion in the various companies.

From the foregoing account, it will be seen that the addition of water to evaporated milk would make it exactly similar to the original milk if none of the constituents were altered or destroyed by the manufacturing processes. These are of such a nature that they would probably not produce marked changes in most of the milk constituents, and would not destroy appreciable amounts of any of the vitamins except B_1 and C. As has already been pointed out, the destruction of B_1 probably does not amount to more than 25 per cent of that originally present; evaporated milk cannot be regarded as an adequate source of vitamin C. More detailed information regarding its nutritive properties is to be found in Sherman's book.[246]

Powdered milk. Milk powder is made from milk by removing the water almost entirely. Either whole milk or skim milk may be used as the raw material for its manufacture. The water is removed, sometimes by spraying the milk against the surface of a heated metal cylinder, and sometimes by running a fine spray of milk into heated air. The water is reduced to about 3 per cent of the total weight of the product, and the other constituents bear the same proportional relations to each other as they do in the original whole or skim milk.

Whole milk powder is in the same class of milk products as evaporated milk, and very much the same comments apply to its nutritive properties. It is a good source of vitamin A, a fair source of the vitamin B complex, and may contain some of the C vitamin. Hart, Steenbock and Ellis [108] find that the roller process of drying milk has very little effect on its content of vitamin C. The very interesting work of Sherman and Campbell [237] in which it was shown that whole milk powder combined

with ground wheat makes an excellent diet for rats and will keep them thriving through many generations, has already been discussed.

The probable nutritive properties of skim milk powder can be inferred from what has been said about those of whole milk powder and of skim milk. There has been some experimental work carried out on its vitamin content which shows, as was to have been expected, that it differs from whole milk powder in the large reduction in the content of vitamin A.[246e] The work of Sherman and MacLeod[238] showing the great inferiority of skim milk powder to whole milk powder as a food for rats has already been discussed.

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Chapter XV

Physiology of Milk Secretion

Introduction

The physiology of milk secretion has been studied from several different aspects by widely different experimental methods. The mammary gland becomes functionally active only at certain periods in the life of the mammalian female, and, at certain stages in the life cycle, it undergoes very marked changes in size. A great deal of experimental work has been done to determine the causes of the growth and changes in functional activity of the mammary gland.

When this gland is fully developed and functioning, on the other hand, many important physiological questions are introduced. A large part of the food eaten by the lactating female is no longer used in her own individual economy, but is converted in her body into a unique kind of nutritive material which for a considerable period in many species will serve as the sole food for the young. This large scale manufacture of food material by the lactating mother necessitates her receiving a diet different from that which is suitable for non-reproducing adults, and, as human beings are every day confronted with the problem of feeding not only the mothers of their own species but also those of their domestic animals, it is not surprising that a very large amount of information bearing on this aspect of the physiology of milk secretion has been collected. In this chapter there will be taken up first the work on the development and functioning of the mammary gland, and later that on the physiology and biochemistry of milk secretion after it is fully established.

Functional Factors Influencing Milk Secretion

Development of the mammary gland prior to parturition. Lactation must be regarded as one of the processes of reproduction. The mammary glands and other reproductive organs are closely associated embryologically and physiologically. The first very considerable development of these glands occurs along with that of the other sex organs and sex characteristics at puberty; and, thereafter, during each successive period of estrus they undergo a more or less pronounced hypertrophy. This is temporary, its extent and persistence depending upon the type of animal, the length of its estral cycle, etc.

That this development of the mammary glands at puberty and the hypertrophy at estrus is brought about by a hormone secreted by the

ovary and carried in the blood is now well established. Puberty and each successive period of estrus are ushered in by certain changes in these organs. Small bodies in them, which are known as "Graafian follicles" and contain the ova, enlarge, protrude from the surface of the ovaries, rupture and release their ova and a fluid that has been formed in them and is known as "liquor folliculi." This process of development of the Graafian follicle is spoken of as its "ripening"; and its release of the ova, etc., as "ovulation." Extirpation of the ovaries in an immature animal prevents the onset of puberty; and, in a mature animal, causes a complete cessation of the estrus. In the former case the mammary glands fail to develop, and in the latter they atrophy. These results are prevented if instead of extirpating the ovaries, they are transplanted to an abnormal position even though their normal nerve supply is completely severed. Further, the condition of puberty may be brought on prematurely or estrus be resumed in mature castrated animals as a result of injecting into them some of the liquor folliculi or extracts prepared therefrom. To complete the chain of evidence, material having an effect similar to the liquor folliculi has been found in the blood immediately preceding and during estrus.

The growth of the mammary glands which takes place in virgin animals in each estral period consists chiefly of a growth of the ducts. In most animals there is practically no growth of the alveolar secreting cells at these periods, and there are no species in which the growth of the alveolar tissue is marked. But, as soon as an animal becomes pregnant, the alveolar tissue begins to grow and this growth continues through the first half of pregnancy. At about the middle of the gestation period, the growth of the alveolar cells stops or becomes retarded, and they begin to secrete a fluid that resembles colostrum. This fluid is retained in the glandular ducts during the latter half of pregnancy; and the increase in size which takes place in the gland during this period is chiefly due to the gradual accumulation of the colostral secretion.

During the last few years it has been discovered that the growth of the mammary alveolar tissue and the initiation of secretion which occur during pregnancy, as well as the estral growth of the duct system, are brought about under the influence of hormones which are formed in other parts of the body. This knowledge has been acquired largely through experiments in which the injection of various glandular extracts into female and even into male castrated animals has brought about marked mammary growth and secretion. An account of the present state of the subject with references to the literature is given in a review edited by Allen,[1] from which the following brief outline is taken.

The *liquor folliculi* from the Graafian follicle described above contains what has been described as the primary ovarian hormone, which has been isolated in crystalline form and given many names, of which "theelin" is perhaps the one most commonly used. Experiments have shown that even in castrated animals injections of theelin are capable of producing a considerable growth of the duct system of the mammary gland accom-

panied by a small growth of the alveoli, and comparable to the mammary development which takes place in normal virgin animals with each succeeding period of estrus. It seems probable, therefore, that the theelin formed in the Graafian follicles which develop in normal virgin animals in connection with each estral period is the cause of the mammary development that takes place at this time.

From the ruptured Graafian follicle there develops in the ovary a body that is known as the "corpus luteum." If pregnancy does not follow ovulation, the corpus luteum does not persist very long, but its life is greatly lengthened when pregnancy and lactation follow ovulation. Extracts have been obtained from the corpus luteum of pregnancy, and have been called "corporin" and "progestin." These extracts injected alone have no influence on mammary development in castrated animals. However, by the use of these extracts in combination with theelin, it has been possible to obtain development of the rudimentary mammary glands of castrated male rabbits in which there was marked growth of both the ducts and lobules of the glands strikingly similar to the development which takes place in the first half of pregnancy in normal females. It seems likely, therefore, that a hormone produced in the corpus luteum of pregnancy plays an important part in the development of the mammary gland which takes place at this period.

The initiation of the mammary secretion which takes place about the middle of pregnancy is brought about by a hormone which can be extracted from the anterior part of the pituitary body. This hormone acts in two ways. It tends to cause the persistence of the corpus luteum which develops from the Graafian follicle, and, in some animals, it has a direct influence on the mammary gland independent of the corpus luteum. In young adult rabbits which have never been pregnant and from which the ovaries have been removed, it is possible to bring about growth and secretion in the mammary gland simply by injections of the pituitary hormone. In rats castrated at estrum, on the other hand, injections of the pituitary extract failed to produce mammary secretion, but this secretion could be induced by treating the rats first with an extract from the corpus luteum and afterward with the pituitary extract.

The foregoing very brief account of the present state of knowledge regarding the hormones which control the growth and secretion of the mammary gland omits many details. The sex hormones have an influence, not only on the mammary gland, but also on other organs and tissues of the body, which is of great physiological importance, but which lies quite outside the scope of this book. Further, different species differ greatly in their response to these hormones as well as in their response to any interference with the normal course of reproduction. All of these details would have to be carefully studied by anyone wishing either to carry on research in the subject or to apply practically the knowledge that has already been obtained.

The remarkable results obtained in this field of physiology suggest, of course, that the sex hormones might be used in a practical way to

increase or prolong milk secretion. So far, however, little or no practical application of this knowledge has been made, and it seems worth while to point out some aspects of the situation which tend to justify the caution that has been observed.

Animals live in good health by maintaining a nice balance among their various functions and activities; and, if any one of these activities is increased, various other changes must take place all through the animal economy in order to maintain the normal balance. For example, the milking function in good dairy cows is already developed to a great extent, probably through a process of human selection in the breeding of these animals. A good dairy cow gives so much milk at the height of lactation that her food intake must be more than doubled in order to support this activity. As things are now, she usually can not eat enough for the first month or so of lactation to support her milk yield, and takes the additional nutriment needed from her own body. If, therefore, milk yield is to be much further increased by hormone stimulation, some means must also be found to increase the capacity of the digestive apparatus. While, therefore, it is probable that further study of the hormones will yield results of great practical as well as general interest, it is, nevertheless, obvious that work in this field is difficult and complicated and is still in the experimental stage.

Secretion of milk prior to and immediately following parturition. Milk secretion is ordinarily regarded as initiated at parturition. This view is not entirely correct. There is some secretory activity normally even in virgin heifers.[6] This is described as serous in nature, opalescent, and containing all the characteristic substances of normal milk. The percentages of casein, fat, and lactose are lower than in normal milk, while those of globulin and albumin are higher. The amount of this secretion is usually exceedingly small, hardly sufficient to permit analysis; but, when removed regularly from the udder, it increases somewhat. Unusual cases are on record of virgin heifers giving as much as ten pounds of milk daily.

The serous secretion characteristic of virgin heifers continues in somewhat increased, but still negligible amounts for several months after they become pregnant. At about the middle of gestation the secretion changes to a thick, syrupy, colorless liquid with a very high protein content characterized by an especially large proportion of globulin and a low content of casein. In the case of a heifer investigated by Asdell this secretion contained 32.2 per cent of protein made up of casein 1.4 per cent, globulin 28.3 per cent, and albumin 1.3 per cent. The secretion had been and continued to be regularly removed from the udder. Before parturition the yield increased to about 12 to 16 pounds, increasing five fold the week before freshening. This contained 4.35 per cent of protein (casein 3.15, globulin 0.43, albumin 0.1), 4.21 per cent of lactose and 3.6 per cent fat. It was in all appearances normal milk. This represents apparently the normal course of secretory activity that may occur before parturition when the milk is removed from the udder. Pregnant heifers in unusual

cases have given as much as 25 to 30 pounds of milk daily for even several months prior to calving. The first secretory products removed from the udder in such cases, according to Woodman and Hammond,[132] appear to be a mixture of normal milk with the globulin secretion noted above. This is also true normally with the first secretion obtained after parturition when the products have not previously been removed from the udder; and these authors suggest that this secretion, known as colostrum, is actually such a mixture of this globulin secretion and true milk.

Although the milk secreted by Asdell's heifer increased five fold during the week prior to calving, it doubled from the day before to the day after this. There is thus a tremendous increase in mammary activity beginning about a week before parturition, and a further large increase just at parturition. The latter is, of course, a matter of common observation, and its cause has been the source of much speculation, but no satisfactory explanation of it has been found. An interesting discussion of this and other points connected with the changes which take place in the mammary gland at various periods in the life cycle of the mammalian female is given by Marshall.[87]

Variation in milk yield with advance in the period of lactation. Milk secretion normally continues to increase for some time after parturition. Turner, Ragsdale and Brody[122] found that, of 80 Holstein-Friesian cows, 40 reached their maximum of 37 pounds daily on an average on the 15th or 16th day after parturition; 32 of them reached an average maximum of 51.5 pounds on the 18th day; while 8 of them reached 83 pounds on the 28th day. The largest monthly milk yield for Holsteins and Guernseys came in the second month after calving; whereas that for Jerseys and "scrubs" came in the first month.

This increase in milk secretion following parturition is a continuation of that preceding this event and is probably largely due to the same factors coming into play or ceasing to depress secretion at that time. In some other animals it has been shown that the growth of the mammary glands and development of secreting tissue continue for some time after parturition. This is probably true, and is frequently stated to be true with the cow, although definite proof of it is difficult to obtain. There is also a period of undereating which, as it is overcome, may contribute to this protracted period of increase in milk yield; but Brody, Turner and Ragsdale[19] do not find that this increase in yield runs parallel with the increased food intake or that it follows closely the improvement in the condition of the animal due to recovery from the effects of parturition. This agrees with the ideas of Eckles and Palmer[31] and with those of other investigators who have shown that this increase in milk yield continues to occur in the cow despite severe underfeeding or particular deficiencies in the diet, and at the expense of the body's own food reserves. This indicates that some factor other than diet is involved, but it should not be interpreted to mean that the extent of this increase in milk yield, which is an important factor in determining the whole lactation yield, is uninfluenced by the diet at this time. This is distinctly not the

case; and Eckles and Palmer have shown that even the composition of the milk is influenced by the nutritive condition of the cow at the onset of this period. It should also be pointed out that the feeding of the cow during gestation may not only build up a store of material that may be used subsequently in making milk constituents, but may also influence the changes which determine the intensity of the milk-secreting impulse or development of the milk-secreting mechanism.

Brody and coworkers [19] have expressed mathematically by means of the equation for a monomolecular chemical reaction the change in milk yield during the period of increase immediately following parturition. Their equation follows:

$$M = B\,(1 - e^{-k_2 t})$$

M is the milk flow at the time t; B is the initial milk flow and e is the base of natural logarithms, 2.71828.

After the month of maximum production the yield of milk steadily declines so that under uniform conditions each month's yield is a constant percentage of that of the preceding month. The rate of this decline varies with individual cows and with the breed. It is affected by age, seasonal changes, state of nutrition, pregnancy, general management, etc. The decline is slower and the duration of lactation longer in dairy breeds. These are often milked for over a year, and if farrow may secrete milk for several years; whereas beef animals may go dry in a few months. Brody and coworkers [18] found that, under favorable conditions, where pregnancy did not intervene, each succeeding month's production after the month of maximum yield is about 94 per cent of that of the preceding one. They expressed this also by means of the mathematical equation for a monomolecular chemical change as follows:

$$M = Ae^{-k_1 t}$$

M is the milk flow as above at the time t, A is the initial milk flow, and k_1 the constant of decline. According to this equation the milk yield declines asymptotically. These authors came to the conclusion that the initial rise and subsequent decline in milk production can be represented as if due to two consecutive monomolecular chemical reactions by combining as follows the two equations above:

$$M = Ae^{-k_1 t} - Be^{-k_2 t}$$

Figure 40 shows the correspondence that they obtained between the observed yields and the yields calculated according to this equation. The curves apparently agree well with the observed yields. These cows were farrow and the conditions throughout the lactation constant.

The results collected by Brody and his collaborators are very valuable in showing the average rates of rise and decline in milk yield which may be expected in good dairy cows under good conditions of feeding and management. It should be pointed out, however, that there are some objections to supposing that the form of the curve obtained by them is

entirely determined by any type of specific chemical reaction going on within the cow. The rate at which milk yield declines in these animals is highly dependent on the manner in which they are fed, and it does not seem very improbable that the curves of milk yield obtained in the investigation under discussion were to some extent determined by the feeding of the cows as well as by the inevitable changes which take place within their bodies with the progress of lactation.

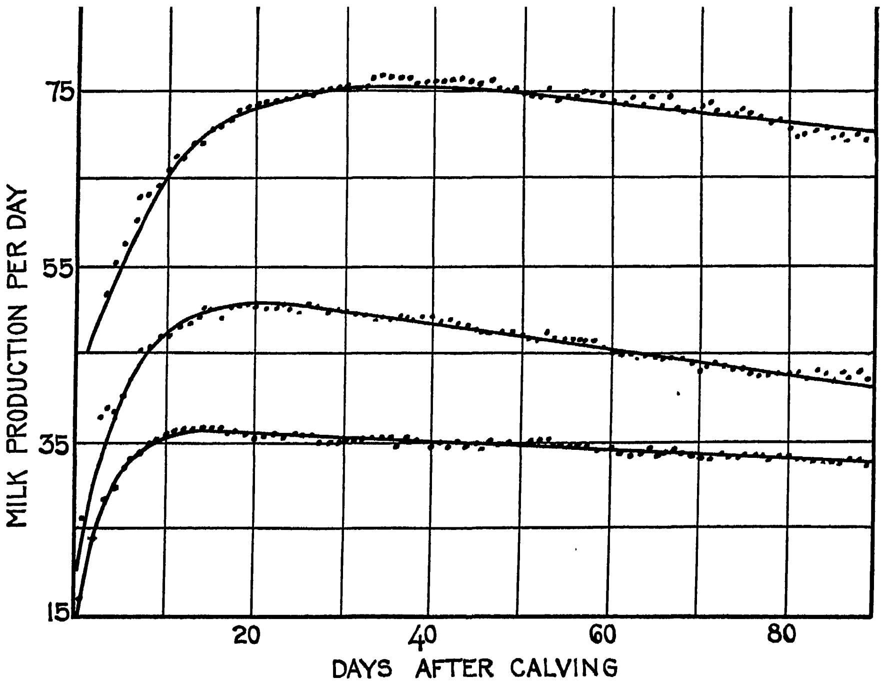

FIG. 40.—The course of milk secretion with the advance of the period of lactation. The dots represent averages from three groups of cows. The lines are plotted from the equation, $M = Ae^{-k_1t} - Be^{-k_2t}$. (Courtesy The Journal of General Physiology.)

Lactation curve as influenced by gestation, castration, and estrus. As indicated above, milk secretion is depressed by gestation. Brody and his collaborators [20] found that this depression becomes significant during the fifth month after breeding, and therefore at the time of accelerated growth of the fetus, and of the mammary glands in the case of the pregnant non-lactating heifer. They found that, with cows in the tenth month of lactation, those that had been pregnant eight months gave about 20 per cent less milk than those that were farrow. They attributed this decline to the nutrients necessary for the development of the uterus, fetus, and fetal appendages. Gaines and Davidson [44] have questioned the adequacy of this explanation, and hold that some hormone with a depres-

sant effect comes into play at this time. These authors have expressed by a mathematical equation the magnitude of the depression in yield brought about by pregnancy. They give factors in Table CXV by which the actual yield of a pregnant cow can be multiplied to obtain the equivalent yield of a farrow cow.

Table CXV.—Correction factors for time calf is carried to convert the milk yield for a whole lactation period to 305-day farrow basis.

P = time in days the calf is carried, from 200 to 280 days

P	0	1	2	3	4	5	6	7	8	9
200.....	1.0015	1.0015	1.0016	1.0017	1.0017	1.0018	1.0019	1.0019	1.0020	1.0021
210.....	1.0022	1.0023	1.0024	1.0025	1.0025	1.0026	1.0027	1.0028	1.0029	1.0030
220.....	1.0031	1.0033	1.0034	1.0035	1.0036	1.0037	1.0039	1.0040	1.0042	1.0044
230.....	1.0045	1.0046	1.0048	1.0050	1.0052	1.0054	1.0056	1.0058	1.0060	1.0062
240.....	1.0065	1.0067	1.0069	1.0072	1.0075	1.0077	1.0080	1.0083	1.0086	1.0089
250.....	1.0093	1.0096	1.0100	1.0104	1.0107	1.0111	1.0115	1.0119	1.0124	1.0128
260.....	1.0133	1.0138	1.0144	1.0149	1.0155	1.0160	1.0166	1.0173	1.0179	1.0185
270.....	1.0192	1.0199	1.0207	1.0215	1.0223	1.0231	1.0240	1.0249	1.0258	1.0268
280.....	1.0278	.....	.....	.....	.....	.....	.....	.....	.....	.....

"Since the correction for pregnancy is of small magnitude, it is permissible first to correct the record for length of time, if different from 305 days, and then to apply the correction of the table to take care of the time the calf is carried."

While pregnancy hastens the decline in milk secretion, castration has been said to retard it. The castration of farm animals, including cows, is a very old practice.[57] Early in the nineteenth century some observations were made which led a number of investigators to believe that the milk yield of cows became more persistent after they were castrated, and the literature from about 1850 to 1900 contains many statements to this effect, backed sometimes by actual figures for the milk yields of castrated cows. A number of authors even recommended that cows should be castrated as a routine dairy practice in order to secure the more persistent milk yield thus made possible.

The earlier experiments on this subject suffer from the circumstance that there is no comparison between the performance of castrated cows and that of cows, which, though still retaining their ovaries are allowed to remain farrow.

The importance of this comparison was, however, recognized by Eloire.[34] He relates the case of a herd, which, owing to the sterility of the bull, failed to become pregnant for a year, and, therefore, continued in milk for a second year. Eloire states that these cows were as persistent in their milk yield as castrated cows had been in previous investigations, but he gives no figures for their performance.

At about the same period Hoffman[67] and Liebener[80] reported comparisons between the persistence in milk yield of castrated and non-castrated cows, giving figures for the results. The latter did somewhat better than the former, but the number of animals studied was too small

to give any final answer to the question. A review of the subject of castration with numerous references to the literature is given by Worch.[133]

The comparisons between the persistence of castrated cows and of non-castrated cows which are kept farrow are still very few, but they indicate that the difference is not great. The same point of view is upheld by a comparison between the whole body of results given for castrated cows and the figures for the normal drop in milk yield of good dairy cows given by Gaines and Davidson.[44] It may be said, therefore, that the evidence at present on hand indicates that castration has no great effect in increasing the persistency of milk yield, and that a very extended investigation with numerous animals would be necessary in order to determine whether or not it has any effect at all.

The effects of estrus and of copulation on the yield and composition of milk have been studied by Stern [118] and by McCandlish.[84] The results indicate that neither estrus nor copulation has any marked consistent effect on either yield or composition, although marked changes may occasionally occur at these periods. When the results from a large number of cows are averaged, it is found that there is a tendency toward a small decrease in milk yield for the days on which they are bred.

Nervous control of secretion. The secretion of milk is an involuntary act. It is quite generally held that it is independent of any true secretory nerve supply from either the peripheral or sympathetic nervous system, but may be influenced by vaso-motor nerves through changes brought about thereby in the blood supply of the udder. Eckhard in 1855,[86] working with goats, showed that the mammary glands are mainly supplied with nerves through a branch from the external spermatic, which originates as a spinal nerve (the second lumbar nerve). The external spermatic nerve gives off branches to the walls of the arteries in the glands, the teats, and the lactiferous ducts. Eckhard found that sectioning this nerve has no effect upon the volume or specific gravity of the milk secreted, and, therefore, concluded that milk secretion was not controlled through the peripheral nervous system. Röhrig,[86] on the contrary, found that cutting the branches of this nerve going to the glands observably retarded, and that electrical stimulation of these nerves brought about acceleration of milk secretion. Further, he found that severing branches of the external spermatic nerve which goes to the blood vessels of the glands led to a 20-fold increase in secretion, and that stimulation of these nerves brought the secretion to a standstill, concluding that there are vaso-motor nerve fibers that influence the secretion of milk. Eckhard criticized the work of Röhrig holding that the latter could not tell whether he was measuring the rate of formation of milk or merely the rate of outpouring of preformed secretory constituents. Laffont and also Valentowicz obtained results agreeing with Röhrig, whereas De Sinety and Partsch agreed with Eckhard.[9]

The interesting work of Goltz and coworkers [9] showed that milk may be secreted independent of the peripheral nervous system. They were able to keep a pregnant dog, in which the spinal cord was sectioned at the

level of the third thoracic vertebra, alive for about two months. During this time healthy young were born, suckled by their mother, and did well. This would exclude the necessity of any true secretory nerve fibers from the central nervous system. It does not exclude the influence of vaso-motor fibers or an effect of the sympathetic nervous system upon secretion. Basch [86] extirpated the coeliac ganglion of the sympathetic nervous system and found it did not affect the volume of secretion, although he did find some changes in the composition of the milk. Probably the changes in milk secretion following nervous disturbances are largely brought about through the effect of vaso-motor nerves upon the blood supply to the gland.

Relation between age and milk secretion. The total yield of milk and milk fat during a lactation period and the shape of the lactation curve vary with the age of the cow. Under proper management the milk and fat yield of a young cow increases from lactation to lactation at a constantly decreasing rate until a maximum production is reached. This is then maintained for several lactation periods and there is then a decline at a rate which increases from lactation to lactation. The age at which a cow attains maximum production varies with the individual, the breed, the feeding and general management. Gowen found that the mean age of maximum productivity with a herd of Jersey cattle was seven years; [49] whereas that of advanced registry Ayrshire, Guernsey,[51] and Holstein-Friesian [50] cows is 10, 9, and 8 years respectively. Graves and Fohrman [53] find that Jerseys reach the age of maximum production at six years and continue at this level of production, other factors being the same, until they reach the mean age of 10½ years. For Guernseys these figures are 5 years and 11 years, respectively. Gowen gives the mean maximum yield with the Jerseys as 37 per cent higher than that at 2 to 3 years of age. With Ayrshire, Guernsey, and Holstein-Friesian cows, these figures were 38 per cent, 25.6 per cent, and 37 per cent respectively. There is a slight irregular tendency for the concentration of milk fat to decline from lactation to lactation; but, since this decline is small compared with the change of milk yield, the variations in the total milk and fat yield run quite closely parallel.

An equation has been devised to fit the curve of change in milk yields with age in dairy cows, but this is quite complicated, involving the use of a number of constants which differ for the different breeds of cows. The matter has been discussed, and tables and equations have been given for the different breeds by Gowen.[49, 51, 52]

Gaines and Davidson [44] found that younger cows are more persistent milkers, and Turner [128] has studied the relation between age and the persistency of fat secretion during the lactation period. He finds that there is a decline in the persistency of secretion during the lactation period as the cow reaches maturity, but that the increase in yield from lactation to lactation is associated with a sufficient increase in maximum yield to more than offset this decline in persistency. He finds the decline to be especially pronounced in passing from the first to the second and third lactation

periods. His data show an increase in maximum monthly fat yield from 40.44 pounds at 2 to 3 years of age to 58.17 pounds at 6 to 7 years.

Structure and physiological relations of the mammary gland. The mammary gland, like other glands, consists essentially of a system of branching tubes which are lined with secreting cells. Over the outer surfaces of these cells run innumerable small blood vessels called capillaries, which bring the blood to every part of the secreting substance of the gland. Through the thin walls of the capillaries various substances diffuse from the blood into the gland cells, and in these cells they are chemically changed into milk, which passes from the inner surfaces of the cells into the tubes and down these tubes to the openings in the teats where it is finally sucked out by the young animal.

The microscopic structure of the mammary gland has been carefully studied, and detailed descriptions of it will be found in any text book of histology. Microscopic studies have suggested to some histologists the theory that milk consists of the entire broken-down bodies of some of the gland cells, the place of these cells thus turned wholly into milk being taken by cells which are provided from the bodies of certain others remaining in place by a process of cell reproduction. Other histologists believe that there is no such rapid destruction and reproduction of cells as this theory would imply, and that each cell simply serves as a little chemical laboratory or factory which takes certain substances from the blood capillaries, converts them into milk, and passes this out through the inner surface into the milk duct.

The mammary gland seldom reaches a size as much as ten per cent of that of the rest of the body, but it is astonishingly effective in producing food which for a considerable period furnishes all that is necessary for the growth of the young animal. From this comparatively small gland there is sometimes secreted in the course of a year ten times as much organic material as is contained in the body of the mother. The materials from which all this milk is manufactured come from the food eaten by the mother; after they have undergone certain chemical changes in her intestinal tract, they are carried thence by the blood to the mammary gland, where they undergo certain other chemical changes before being secreted as milk. There is every reason to think that the digestive processes and the chemical changes which go on in the body generally are very much the same in milking animals as in animals which are not milking. The physiology of milk secretion, therefore, is intimately connected with the general physiology of the body, and can not be considered or understood except as a part of general physiology. It is worth while to remind the reader of certain physiological relations which have an important bearing on the problems of milk secretion.

Manner in which food products are carried to the mammary gland and other organs. Food products leaving the intestinal tract are carried by the blood first to the liver, and are more or less changed chemically in passing through that organ. The liver has some part in adjusting the composition of the blood to the demands which are to be made on its

content of nutritive material by the rest of the body. In milking animals, the demand for nutritive material from the blood is often much more than twice as great as in the same animals when they are not milking; and it is not known how far the liver adjusts its activities to the nutritive demands of milking animals.

From the liver the blood carries the nutritive material which it has picked up from the intestine to the right auricle and the right ventricle. Here, the particular fraction of blood which has come from the intestine is thoroughly mixed with blood coming from all other parts of the body and sent out to the lungs to receive oxygen and to get rid of carbon dioxide. It comes back from the lungs (thoroughly mixed) to the left auricle and ventricle, and is distributed impartially to the mammary gland, and to all other organs and tissues.

All this means that the mammary gland can not receive a particular kind of blood suited to its own peculiar and very large needs for nutritive material. It receives just the same kind of blood as all the other organs of the body, it must compete with the other organs for its supply of nutritive material, and, if the blood is made richer than usual for milking animals by heavy feeding in order to supply the heavy nutritive demands of the mammary gland, all the other organs will have an opportunity to take their share of the surplus.

On the other hand, it is quite conceivable that the mammary gland might compete so effectively with the other organs for nutriment, as to leave them worse off after it had come into action than they were before, even though the milking animal might eat large quantities of food. In later sections experiments will be described which show that these theoretical possibilities do actually occur. In the early stages of lactation the mammary gland often takes nutriment from the blood so vigorously that the milking animal can not eat enough to supply both it and the other organs with what they need, even when given as much food as it will eat. Under these circumstances certain organs and tissues of the body such as the muscles and the fat deposits begin to pour their own substance into the blood. The mammary gland uses this material from other parts of the body for the manufacture of milk, and the animal thus secretes milk at the expense of its own body tissue. There is reason to think, however, that, when this occurs, the milk flow is practically always reduced faster than it otherwise would be.

Toward the end of the period of lactation, the mammary gland becomes less able to take nutritive material from the blood in the face of a general shortage. As a consequence, the milk flow falls off rapidly unless enough food is supplied to keep the blood in such a stage that the muscle and fat deposits, as well as the mammary gland, can take nutritive material from it.

Food Factors and Milk Secretion

Changes undergone by the food during digestion. The food eaten by mammals is subjected in their digestive tract to mechanical and

chemical changes which get it into solution in the digestive juices. In the mouth it is ground up by the teeth, soaked with the saliva, and brought into contact with ptyalin, the first of the digestive ferments, which is contained in the saliva and which has the effect of converting the starches of the food into the more soluble sugars. From the mouth it is passed on through the digestive tract by means of the muscular movements of the tubular walls, and, during the process, it is continually kneaded and mixed until it becomes a more or less uniform thick fluid. At various stages of its progress, it encounters and is mixed with various digestive ferments and chemical substances. The stomach secretes hydrochloric acid and pepsin, which convert the food proteins into simpler and more soluble intermediate products called proteoses and peptones. Just below the stomach, the pancreas pours alkaline sodium carbonate into the intestine, accompanied by three powerful ferments which carry farther the breaking up of proteins into peptones, of starches into sugars, and of neutral fats into glycerine and fatty acids. Along with the pancreatic secretion comes bile from the liver, which aids in the digestion and absorption of fats. Still further on in their progress, the food products encounter erepsin, a powerful ferment secreted by the intestinal walls, which carries the digestion of protein all the way down to the decidedly simpler and more soluble amino acids.

Course of the fully digested food products in the body and the chemical changes which they undergo after they leave the intestinal tract. The foods, having been chemically changed and rendered soluble by the digestive activities, pass through the walls of the intestinal tract and enter the blood stream. In order that this may occur, the proteins are converted to amino acids, the carbohydrates to simple monosaccharide sugars, and the fats to glycerine and soaps. All of these products except the glycerine and soaps are taken by the portal vein directly from the intestinal tract to the liver, where some of them undergo further chemical transformations, and then on by the hepatic vein into the general circulation. The glycerine and soaps are reconverted to neutral fats as they pass through the intestinal wall. They are taken up through small tubular vessels called lacteals into the lymphatic system, and thrown into the general circulation through the thoracic duct, thus avoiding the direct transportation through the portal vein to the liver, which is undergone by the products of protein and carbohydrate digestion. The monosaccharides which pass from the intestine to the liver through the portal vein are converted in the liver to dextrose, if they have not already assumed that chemical form. All of the products of the digestion of protein, fat, and carbohydrate, therefore, are finally converted to amino acids, neutral fats, and dextrose, and are carried about by the blood in these three forms, and thus distributed to the various organs and tissues.

They are taken up by the organs and tissues, and in them undergo further chemical transformations. Under appropriate circumstances they may be built back into more complicated chemical bodies like those from which they came—the amino acids into protein, and the dextrose into

glycogen. But the great majority of them undergo further breakdown and oxidation. The amino acids are de-aminized and converted into urea, carbohydrate, and probably also fatty acids. The dextrose and neutral fats are oxidized, converted into various little-known intermediate bodies, and finally into carbon dioxide and water. The carbohydrate and possible fatty acids derived from protein undergo similar changes so that the chief end products of metabolism are carbon dioxide, water, and urea. The energy which is released in the transformation of amino acids, dextrose, and fatty acids to carbon dioxide, water, and urea may appear simply as heat, or it may be used to supply the energy required for muscular activity.

Much study has been given in the last 30 years to the question as to which constituents of the blood are used by the mammary gland for the manufacture of the milk constituents. In 1906 Kaufmann and Magne compared the dextrose content of samples of blood obtained respectively from the jugular and mammary veins of milking animals and found that the dextrose was always less in quantity in the mammary than in the jugular blood. They concluded from these observations that the mammary gland used dextrose, which it took from the blood, for the manufacture of milk sugar.[78] Several years later, Meigs, Blatherwick and Cary [80] and Cary [22] studied the phosphatid and amino nitrogen in jugular and mammary vein blood, and concluded from their results that milk fat is manufactured from the phosphatid of the blood, and milk protein from the free amino acids and short-chain polypeptides. Still later, Blackwood and Stirling [13, 14, 15, 16] subjected the previous work along these lines to a careful review and criticism. They compared the jugular vein blood with the arterial blood in cattle and found that the corpuscles and iron were more concentrated in the former. They give reasons for believing that this is due to the large amount of water taken from the jugular vein blood by the salivary glands. The jugular vein blood is, therefore, not in all respects the same as the arterial blood going to the mammary gland, and the conclusions of Kaufmann and Magne, and of Meigs, Blatherwick and Cary, cannot be regarded as valid unless it can be shown that the dextrose, phosphatid and amino nitrogen are less in the mammary vein blood than in the arterial blood of milking animals. Blackwood and Stirling then compared the dextrose and amino nitrogen in these two kinds of blood and found that the quantities of both these constituents were less in the mammary vein blood. The conclusions of Kaufmann and Magne, and of Cary, in regard to these two constituents were thus found to be correct.

Very recently, Lintzel [82] has studied the phosphatid and triglycerides in the arterial and mammary vein blood of milking animals, and also the dextrose and amino nitrogen. He finds that the dextrose, amino nitrogen and triglycerides were all reduced in quantity in the mammary vein blood, while the phosphatid has about the same concentration in the two kinds of blood. The conclusions drawn by Meigs, Blatherwick, and Cary from their study of the phosphatid in jugular and mammary vein blood are

therefore not correct, and milk fat is manufactured from the triglycerides of the blood.

There is every reason to think, therefore, that, under ordinary circumstances, milk protein is made from food protein, milk fat from food fat, and milk carbohydrate from food carbohydrate. But it has also been clearly shown that this is not always the case, and the numerous other possible ways in which the mammary gland may get materials for the manufacture of its product must be kept clearly in mind in order to interpret the results of the numerous experiments which have been carried out to determine the effects of changes in the food supply on milk secretion.

A review of the work on the relation between milk secretion and diet and a fairly extensive bibliography has been published by Meigs.[91] In this review the manner in which certain important physiological principles rest on experimental work has been taken up in some detail, and it will not be necessary to repeat this discussion in the following treatment. On the contrary, the effort will be made to give as concise as possible a statement of the points which may be regarded as established and to refer only to the outstanding pieces of work on the subject. For a more complete bibliography the reader is referred to the review above mentioned.

Effects on milk production of deficiencies in the food supply. If food is withheld from a milking animal altogether, milk secretion does not immediately stop. The animal pours into the blood from all parts of the body the amino acids, dextrose, fats, mineral salts, and other materials which are necessary for milk secretion, and the mammary gland uses these materials from other parts of its body for the manufacture of milk. All of these constituents which are necessary for milk secretion may, therefore, be supplied to the blood of a milking animal from parts of its body other than the intestinal tract, and this fact will be found again and again to play a part in the interpretation of the results obtained in experiments on the nutrition of milking animals.

Instead of being deprived of food altogether, a milking animal may be fed a ration which contains plenty of some of the constituents necessary for milk secretion, but is deficient in others. Under these circumstances two possibilities arise. In some cases, the animal may manufacture the missing constituents from other constituents of the food. It has been shown that milking animals can make milk fat and milk carbohydrate from food protein, and that they can make milk fat from food carbohydrate. In other cases, the milking animal can not make the missing constituents from other constituents of the food, and takes them from her own body.

The effects on milk yield of withholding certain dietary constituents has been quite extensively studied. Although the milk yield does not immediately cease, even when all food is withheld, it is, nevertheless, an invariable rule that the withholding of any single essential constituent or of any group of essential constituents soon results in a more rapid drop

in milk yield than occurs under adequate dietary conditions. The extent to which milking animals will rob their bodies for the benefit of their milk yield is different according to the dietary constituent involved. The withholding of certain constituents from the food results in a very rapid drop in milk yield and a comparatively small loss of body substance; in other cases the milking animal may go on for a long time robbing her own body and showing a drop in milk yield not very markedly more rapid than that which occurs under optimum dietary conditions. The effects on the yield and composition of milk of making various particular changes in the diet will be taken up in the following sections. The question of the relation between nutritive energy and milk yield has been more extensively studied than any other part of the subject, and will be taken up first.

Effects of changes in the nutritive energy of the food on milk yield, and the economy with which the energy of the food can be converted into milk energy. It is a matter of common observation that milking animals which do not receive enough food fall off rapidly in milk yield and lose weight. They use their body fat and some of the protein from their muscles to supply the nutritive energy which is needed for their milk, but this use of body substance does not prevent a rapid drop in milk yield, if the food shortage be at all severe. It has been shown by Eckles and Palmer [31] that the effects of food shortage are different according to the stage of the lactation period in which they occur. Cows fed insufficient rations in the early stages of lactation show a remarkable power of maintaining a fairly steady milk yield in the face of a rapid loss in body weight, while later the food shortage has more effect on milk yield and less on body weight. It is evident that, in the early stages of lactation, the mammary gland competes more effectively with the other tissues for the supply of nutriment that is carried in the blood than it does later on. It is very important to keep these facts in mind in considering the experimental work which has been carried out to determine how much nutritive energy is necessary to support a given milk yield.

Before taking up the experimental work on the relation between food energy and milk yield, it will be worth while to consider some of the theoretical aspects of the subject. As has already been pointed out, there is reason to believe that food protein is converted to milk protein through a change to free amino acids and back again; that food carbohydrate is converted to glucose, and then to lactose; and that food fat is converted to glycerine and fatty acids, and then again to neutral fat. As far as is known, all these chemical changes might go on with very little expenditure of energy.

A mammal, of course, needs a certain amount of nutritive energy to maintain it—to keep its body temperature somewhat above the external temperature and to supply the energy necessary for the activity of its muscles and glands. In the experimental work on the energy required for milk secretion this energy required for maintenance has always been taken into account. The usual procedure has been to determine the

maintenance requirement in animals which were not milking, and later to study the total food requirements of the same or similar animals when they were milking. The proportion of the nutritive energy of the food which is utilized to produce the nutritive energy of the milk is calculated by subtracting from the total food intake of the milking animal the energy required for her maintenance as determined in the first part of the experiment and then comparing the remainder with the energy contained in the milk.

It is obvious that, as far as chemical considerations go, a very large proportion, if not all, of the food energy furnished to a milking animal over and above the maintenance requirement might be converted into milk energy. Even though some of the food constituents could not be wholly converted into the corresponding constituents of milk, there is, nevertheless, no reason why the fractions not utilizable for milk production should not be used to supply a part of the energy required for maintenance. For example, the vegetable proteins which are usually contained in the food of cows do not have the same amino acid complex as the milk proteins, and can not be converted gram for gram into milk protein. But there is no reason why the energy contained in the amino acids not utilizable for the manufacture of milk protein might not be utilized for the maintenance processes, and thus escape the appearance of being wasted in the results of an experiment calculated as above outlined.

In spite of the many attractive reasons for thinking that the nutritive energy furnished to a milking animal over and above her maintenance requirement might be wholly converted into milk energy, the experimental work on the subject has never shown that milk secretion goes on as economically as this. In a few experiments about 70 per cent of the food energy over and above the maintenance requirement has been converted into milk energy, but in the great majority of cases the economy of milk production has been definitely lower than this and rather variable. A consideration of the anatomical and physiological relations between the mammary gland and the rest of the body will show that this outcome of the experimental work is not really very surprising.

As has already been pointed out, the mammary gland is situated on a shunt of the circulation, and must compete with the other tissues for the nutriment contained in the blood. The economy with which an animal can secrete milk will therefore depend, for one thing, on the effectiveness with which her mammary gland can compete for nutriment with the other tissues, and, for another, on how economical in the utilization of energy her tissues happen to be when the nutritive material contained in the blood is at any given level. It is a physiological commonplace that the tissues of an animal adjust their energy output more or less to the supply of nutritive energy contained in its blood. If, therefore, the mammary gland can compete very effectively with the other tissues for nutriment and go on secreting milk at a rapid rate when the supply of nutritive material in the blood is low, it will soon force the other tissues to adjust themselves to the low level of blood nutriment at which it can work, and

the animal as a whole will secrete milk economically. But if the mammary gland can not compete so effectively with the other tissues, the rate of milk secretion will be rapidly reduced unless the nutritive material in the blood is maintained at a level high enough to permit them to be somewhat wasteful, and the animal as a whole will secrete milk less economically. It is well known that different individual animals differ in the economy of their general use of nutritive energy, and the work of Eckles and Palmer [81] above referred to shows that the mammary gland competes more effectively with the other tissues for nutriment in the early stages of lactation, and less effectively later. It is to be expected, therefore, that the economy of milk secretion should vary, not only from individual to individual, but also in the same individual at different times.

Kellner [74] has carried out experiments in which he made complete determinations of the energy intake and output of three milking cows in order to arrive at the efficiency of conversion of food energy into milk energy. The experiment with each cow lasted for two weeks. He calculated the energy required for the maintenance of these cows or for any gain or loss of protein or fat as pound for pound the same as in the case of oxen, with which he had previously made determinations of this sort. Deducting, therefore, from the total energy intake in the food, that lost in the feces and urine, and that utilized in maintenance and loss or gain of body tissue, he arrived at the amount of energy available for milk secretion and compared it with that actually occurring in the milk. The latter was 68.4, 72.8, and 66.9 per cent, respectively, of the former. On an average 69.4 per cent of the energy available for milk secretion occurred in the milk.[5]

Eckles [80] carried out determinations of this sort in an entirely different way. He followed the food intake, milk and fat yields, and body weight with eight milking cows for a year each and with two others for 110 and 120 days respectively. In the same cows the maintenance requirement was determined when they were dry. In all cases the food was so adjusted as to maintain constant body weight. Digestion experiments were run both when the cows were milking and when they were dry. Armsby [5] made use of the data obtained by Eckles in order to determine the economy of his cows. Calculating the total metabolizable energy in the food absorbed and deducting that for maintenance, he arrived at the amount of energy available for milk secretion and compared it with that in the milk. Using the calculation of the metabolizable energy in the food in accord with data given by Armsby for this purpose, the actual energy in the milk varied from 50.4 to 72.8 per cent of that available for milk secretion, or an average of 61.9 per cent. Haecker obtained data which when similarly calculated gave an average figure of 54.7 per cent.[5]

More recently, Meigs and Converse [98] have carried out experiments more or less similar to those of Haecker and Eckles. Their results are in close agreement with the earlier ones in regard to the amounts of nutritive energy required to keep milking cows in nutritive equilibrium.

The results of Kellner are not really in wide disagreement with those

of Haecker and Eckles and of Meigs and Converse, as far as can be judged from the material at present at hand. Unfortunately the results of Kellner have never been fully published; all the information that can be obtained on the subject at present is contained in the short article above referred to in which the figures obtained with three of the experimental animals are given, and show an economy in the sense above outlined of about 70 per cent. But the author states that a number of other animals were found to be considerably less economical than this. It seems justifiable to sum up the results of all four investigations by saying that they indicate that the proportion of nutritive energy over and above the maintenance requirement which cows convert to the energy of milk varies, and usually lies between 55 and 70 per cent.

The practical conclusions which have been drawn from the experimental results above outlined have varied widely and have given rise to a good deal of controversy. The reader who wishes to follow the matter in more detail is referred to Armsby's book [5] and to articles by Meigs and Converse,[93, 94] and by Forbes.[42] The present discussion may be closed by pointing out one or two additional aspects of the subject which can hardly be regarded as controversial.

In considering the question of the economy of milk production, it must be remembered that the phrase can be used in two sharply different senses. In the experiments of Kellner above referred to, the economy of production is measured by subtracting the energy required for maintenance from that of the total food intake and comparing the remainder with the energy contained in the milk. If the energy of the milk be called E; that of the total food intake, F; and that required for maintenance, M; the economy in this sense is measured by the fraction $\frac{E}{F-M}$. This may be called the special economy of production. But the milk producer is more interested in the simple relation between the milk production and the food intake, which is measured by the fraction $\frac{E}{F}$, and may be called the general economy of production. The value of this fraction depends very largely on the level of milk production and there is, therefore, no necessary parallelism between it and the figure for the special economy of production. Suppose, for instance, that at the beginning of lactation $E = 7$, $M = 10$, and $F = 20$; while, at the end, E is reduced to 3, and F to 14, M, of course, remaining constant. Then at the beginning of lactation, the general economy of production will be 0.35, and the special economy 0.70; while, at the end, the general economy will be only 0.214, although the special economy has risen to 0.75. What this means from the practical point of view is that it is quite as important for the milk producer to maintain a high yield in his animals as to cause them to produce with the greatest possible special economy, and, in considering experimental work on the economy of milk yield, he must necessarily be as much interested in the effect which the experimental procedure had on the maintenance of yield as in the exact degree of special economy

attained. In experiments such as those of Kellner [74] which last for only a few weeks, it is not possible to get much light on the effect of the procedure on milk yield. The effect of various levels of energy in the feed on the maintenance of milk yield varies, as has already been pointed out,[31] according to the stage of lactation. The experiments of Kellner, therefore, in spite of their technical perfection, fail to throw light on a point which is of primary importance from the practical point of view, and which is very well covered in the less elaborate experiments of Eckles,[30] namely, the effect of the experimental procedure on the level of milk yield when continued through a whole lactation period.

There has been only a small amount of experimental work done on the effects of different levels of energy in the diet on the maintenance of milk yield. Cary and Meigs [28] found that large reduction in the energy content of the diet produced sharp reductions in milk yield, and that the milk was to some extent restored when the dietary energy was put back to its original level. Converse [24] studied the effect of supplying milking cows through whole lactation periods with somewhat more nutritive energy than Eckles [30] found to be necessary to maintain uniform body weight. The animals so fed showed a noticeably less rapid drop in milk yield than when fed at the same level as those in Eckles' experiments. It seems very unlikely, therefore, that feeding cows less nutritive energy than Eckles found necessary to maintain uniform body weight would result in any long continued general economy in milk production.

Effects of changes in dietary carbohydrate on the composition of milk. Most of the nutritive energy in the rations of dairy cows is supplied in the form of carbohydrate; and changes in dietary carbohydrate therefore involve parallel changes in nutritive energy. The effects on milk secretion of changing the dietary carbohydrate have not been thoroughly studied. Small changes in this dietary constituent have little effect on either the composition or the quantity of milk secreted.[91] Large reductions, on the other hand, cause a rapid falling off in milk yield. The concentrations of fat and lactose in the milk remain unchanged, while that of the nitrogen is somewhat reduced.[28] This latter change is of great physiological interest and will be discussed more fully under the next heading. The constancy of the lactose concentration in milk is also very interesting. Numerous investigations show that this constituent remains nearly constant in percentage in spite of a great variety of changes in the diet of the milking animal.[91] Work of Cary and Meigs [28] shows that the variation of this constituent in the milk of individual animals under widely varying diets is no wider than in the ratio of 100/104.*

Effects of changes in dietary protein on milk secretion. The study of the relation of protein in the diet to the secretion of milk has been attacked with the following objects in view:

* In order to avoid confusion of various kinds which result from expressing variations in the percentage concentrations of the milk constituents as percentages, this method of expression will be used throughout this discussion. The numerator of the fraction represents the lower limit of the variation; the denominator, the upper limit. The above ratio, for example, represents variations in milk constituents from 5.0 to 5.2 per cent, or from 0.100 to 0.104, etc.

1. To determine the relative efficiency with which various feed proteins may be utilized by milking animals.

2. To determine from a practical and economic standpoint the most profitable level at which to feed protein to milking farm animals.

3. To determine the character of the changes in the yield and composition of milk produced by changes in the quantity or quality of protein in the ration, and the changes in the blood by which these are brought about.

The problem of the conversion of food protein into milk protein presents many of the same features as that of the conversion of food energy into milk energy. On account of its situation on a shunt of the circulation, the mammary gland must compete with the other tissues for the protein products in the blood, just as it must compete for other food products, and the general economy of protein utilization will depend on how effectively it can carry on this competition. But the problem of protein utilization is experimentally much more complex and difficult than that of energy utilization. In the case of the latter problem, heat production serves as a common measure to which all forms of dietary energy can be converted, but the protein problem is multiplied into the numerous separate problems of the essential amino acids. If a single one of these is absent from the diet, the dietary protein alone becomes entirely useless as such as raw material for milk production. Strictly speaking, therefore, it is impossible to give any figures to represent the economy of protein utilization in milk secretion. The economy of utilization will depend on the kind of dietary protein supplied.

There are other circumstances which complicate the study of the utilization of food protein in milk secretion, some of which have already been discussed in the preceding chapter. A further discussion of the subject will be found in the article by Cary and Meigs [28] already referred to. In view of all these difficulties, it is not surprising that the experimental results obtained in the field are often apparently somewhat contradictory and difficult to reconcile with each other.

But, in spite of the difficulties of the subject, some progress has been made toward getting a rough practical estimate of how much food protein is necessary to produce a given amount of milk protein under ordinary conditions, toward discovering some of the effects of changes in dietary protein on milk yield, and even toward some understanding of how dietary changes affect milk yield through changes in the composition of the blood. The work bearing on these questions will be outlined in the following pages.

Hart and Humphrey [61] have compared the efficiency of utilization of the proteins of various feeds when fed to milking cows. They aimed to vary only the kind of protein in the diet, and to keep its amount and the energy content of the diet constant. The food N minus the fecal N was taken as the N absorbed. The utilization was calculated as the sum of the milk N and the N balance.

This, expressed as percentage of the absorbed N, was taken as a

measure of the efficiency of the protein of the feed for milk production. Milk protein itself when fed, thus gave an average efficiency of 59 per cent as compared with 36 per cent and 40 per cent for wheat and corn proteins respectively. This calculation allowed nothing for maintenance. If, following the conventional procedure, 0.5 pound of absorbed protein per 1,000 pounds of live weight be allowed for this purpose (and the weights of these animals be taken as 1,000 pounds), approximately 100 per cent of the protein calculated as available for milk secretion would occur in the milk on the milk protein diet. The corresponding figures for wheat and corn proteins would be 60 per cent and 67 per cent respectively. But it is obvious that the same amounts of these three different kinds of protein should not be allowed for maintenance; that there is no way of estimating the amounts that should be allowed; that each allowance would give a different series of results; and that, regardless of whether this correction for maintenance could be correctly made or not, constants would not be obtained that would be characteristic of the feed protein itself. These facts were fully appreciated by Hart and Humphrey, when they carried out this excellent work.

On the other hand, the results of Hart and Humphrey, cited above, leave little doubt of the more efficient utilization of milk proteins than of either wheat or corn proteins by a milking cow. The variation of their results with wheat protein from 23.1 per cent with one cow to 49.1 per cent with the other, indicates that the difference between the average values obtained for it and corn protein is not significant. They made similar determinations with numerous other proteins and protein mixtures. For these the reader is referred to their original articles.[61]

In judging the criteria often presented regarding the adequacy of the protein in the diet of a milking cow, it is of interest to note in these experiments of Hart and Humphrey that when they changed from feeding a more efficient protein to a less efficient one the amount of milk protein secretion was frequently not reduced. That the diet was thereby rendered inadequate in these experiments and that the secretion of milk was maintained at the expense of the proteins in the tissues of the animal, are indicated by the change of the N balance from positive to negative with the change of diet. In some of their experiments, the milk yield was thus maintained for 8 to 12 weeks, despite a continuous negative N balance, before a drop set in that was definitely greater than might be expected with the advance in lactation. There was then a decided drop in the secretion of milk.

Numerous other experiments have been carried out to determine the effect on the yield and composition of milk of changing the protein of the diet. In very few of these with cows have the changes involved only the dietary protein. In general these changes have been much more complicated; and the results in many respects have been correspondingly variable. There is, however, a general agreement in all of them that the quantity of milk yielded is highly dependent upon the quantity of protein supplied in the food. It is a very general rule that increased protein in

the food results in an increased milk yield, and vice versa. Morgen and collaborators,[100] working with sheep and goats, found that additions of protein to the ration at almost any level tend to increase the milk yield; and so true is this with cows that many authorities attribute to protein a general stimulatory effect upon the secretion of milk.

Cary and Meigs [23] studied the changes in the yield and composition of milk brought about by large changes in the quantity of protein in the ration of dairy cows. The changes were made abruptly. Three animals were first put upon diets which, according to the ordinary feeding standards (they were fed approximately in accord with the Savage standard for feeding milking cows. See Henry and Morrison, p. 133 [66]), were adequate in every respect for their maintenance and production. The amounts of dietary protein were later reduced about 30 per cent, and subsequently this change was reversed. It was found that these decreases in the amounts of protein in the ration immediately reduced the yields of milk. In five days these reductions amounted to about 15, 17, and 22 per cent respectively, and one was as much as 37 per cent in 15 days. At the same time the concentrations of protein and fat in the milk were reduced. The concentration of lactose in the milk remained constant. These changes in the yield and composition of milk were reversed when the dietary protein was again increased. In this work these investigators came to the conclusion that the changes in the quantity of protein in the ration affected the secretion of milk mainly through changes in the composition of the mixture of amino acids in the plasma of the blood, and demonstrated the occurrence of such changes by determining simultaneously the concentrations of total amino acid N and of protein-free tryptophane in the blood at intervals before and after the change of diet. So great and so effective are these changes in the composition of the amino acid mixture in the plasma of the blood that, although under certain conditions an increase of 100 per cent in the quantity of protein in the ration may lead to a drop in the total plasma amino N, the yield of milk, and the concentration of milk N may nevertheless be decidedly increased.

These workers also carried out experiments in which they varied the kind of protein in the diet, the amounts of energy and protein in the ration being kept constant as in the experiments of Hart and Humphrey. The cows studied by Cary and Meigs were fed amounts of protein approximately in accord with the Savage standard (see above). When the dietary protein was changed from a mixture of milk and corn proteins to corn protein alone, or from corn proteins to a mixture of corn protein and gelatin, the milk yield was immediately reduced. In the latter instance it dropped 25 per cent in ten days; but was increased 24 per cent in the next ten days as a result of a change from a mixture of corn and gelatin proteins to one of corn and milk proteins.

These experiments, as well as those of Hart and Humphrey, show clearly that the quantity of protein that it is necessary to feed to maintain a given milk yield depends upon the kind of protein in the diet. These two sets of experiments differ simply in the time that elapsed before the

yield of milk responded to the change of diet. This probably depended mainly upon the N reserves of the animals used and the vigor of the milk secreting impulse at the stage of lactation at which the experiments were carried out.

Cary and Meigs found that, under suitable conditions, it may be demonstrated that there is a tendency with a milking cow for the total amino N of the blood and blood plasma to be lower when the composition of the dietary protein is such that it can be more efficiently used to meet the animal's N requirements. On the other hand, the milk secretion is increased under these conditions. These workers attributed this effect of the change of diet on the secretion of milk to changes in the composition of the blood mixture of amino acids, which they found to occur just as in the case of changes in the quantity of protein in the ration.

Cary and Meigs found also that the amino nitrogen of the blood and the protein concentration in the milk were affected, not only by changes in the dietary protein, but also by changes in dietary energy. Large reductions in the dietary carbohydrate reduced the amino nitrogen of the blood plasma more than they did the carbohydrate, and reduced the protein of the milk more than they did its carbohydrate. The milk yield was also much reduced under these circumstances. There is reason to think, therefore, that the amino nitrogen of the blood plasma is a central factor in controlling milk secretion, and that it can be acted on by changes, not only in the protein, but also in the energy of the food.

The concentration of protein in milk is less constant than that of the lactose, but still quite constant. In the experiments of Cary and Meigs, its variation was 100/125 in the milk of individual animals.

From the results cited above, it is apparent that the secretion of milk is more readily responsive to changes in the protein of the diet than to proportionate changes in other dietary constituents, and it is worth while to try to sum up somewhat comprehensively the knowledge that has been obtained regarding the effects of dietary protein on milk yield. The terms general economy and special economy of protein utilization will be used in the same sense as in the discussion of energy utilization.

Animals adjust their utilization of protein to the supply in the food, just as in the case of other food factors. Suppose that a milking animal is first fed enough to maintain her in protein equilibrium at a certain level of milk yield, and that the food protein is then reduced. The N balance will immediately become negative, and, at the same time, there will begin a gradual reduction in the use of protein by the general body tissues and a gradual reduction in the milk yield. After an interval the animal will come again into N equilibrium at a lower level of milk yield and with a greater economy in the use of protein by the general body tissues. The special economy of protein utilization will be greater at the lower level of milk yield, but whether or not the general economy will be greater will depend on how much the milk yield has been reduced.

The extent to which reductions in dietary protein affect the milk yield varies greatly with a number of circumstances. In high producing ani-

mals carrying a large surplus of body protein in the early stages of lactation, reductions in dietary protein have their chief immediate effect on the N balance, and the milk yield is less noticeably affected; under opposite conditions in all three respects, the reduction in milk yield in response to a reduction in dietary protein is more rapid. The problem is so complicated as to be hardly soluble in the quantitative sense.

Standards which give recommendations as to the amounts of protein that should be fed to support given milk and fat yields in dairy cows have been formulated by Haecker [56] and Eckles.[80] But an examination of the experimental work on which these standards are based shows that there was no attempt to determine the effects of different levels of protein feeding on milk secretion. The results are interesting and valuable in showing that under certain circumstances a certain amount of milk and fat could be produced at a certain level of protein feeding, but they do not throw much light on the question whether that particular level was the most economical in any sense.

Savage [112] compared the amounts of milk and fat yielded at the level of protein feeding recommended by Haecker with those yielded at a higher level. His results indicated that the higher level of protein feeding induced a higher milk and fat yield, and he has formulated a standard recommending a higher level of protein feeding than did Haecker.

There have been a great many short-period experiments in which the effects on milk yield of changing the dietary protein have been studied. This work has been reviewed by Meigs [91] and further information on the subject will be found in the article by Cary and Meigs [23] already frequently referred to. The results may be summed up by saying that increases in the dietary protein have a strong tendency to increase the milk yield; that decreases have the opposite effect; and that the effects of changes in the dietary protein on the composition of the milk have been rather small and very variable.

The state of the subject as outlined above is perhaps not very satisfactory for the guidance of the practical milk producer. It should be recognized at once that no definite quantitative recommendations can be given for feeding protein so as to produce milk with the highest economy. But the practical producer is, nevertheless, somewhat better off than if no work at all had been done on the subject. The work of Haecker, Eckles, and Savage shows that, under ordinary circumstances, milk can be fairly economically produced at certain definite levels of protein feeding. The short-period experiments above referred to, on the other hand, indicate that milk production can often be driven far above the usual level by feeding even more protein than is recommended by the Savage standard, and that, where high production rather than strict economy is the chief goal, it is advisable to feed protein very liberally. Most of the dairy herds in this country which are fed for high production receive far more protein in their rations than is recommended by any of the feeding standards.

Relation between milk secretion and the metabolism of fat. The problem of the secretion of milk fat is simpler in some respects than that of the secretion of milk protein, but it should be remembered, in beginning the consideration of the subject, that, whereas milk protein can be derived only from the protein of the food, milk fat can be derived from either dietary fat, protein or carbohydrate. Very convincing evidence for the view that milk fat is commonly derived from food fat, however, is afforded by the numerous experiments in which it has been shown that the composition of the milk fat is influenced by that of the food fat, and even that certain fatty acids not ordinarily present in milk fat may be made to appear there by supplying them in the food. These experiments will be more fully considered later, while the experiments in which the effect on milk secretion of changing the quantity of fat in the food has been studied will be considered immediately. The results are in general accord with the view that animals can produce milk fat more readily from food fat than from food protein or carbohydrate, but that the two latter classes of foods can be easily utilized for fat production when the supply of food fat is low.

Morgen and his collaborators [97, 98, 99] working with sheep and goats, studied the effect on milk yield of varying the quantity of fat in the ration. They found that when the quantity of fat fed to these animals was less than 0.5 gram daily per kilogram of body weight, adding fat to the ration markedly increased the percentage of fat in the milk as well as the total milk yield. This was true until the fat in the ration had been increased up to about one gram per kilogram of body weight. The further substitution of fat for carbohydrate was then without avail. When the milk yield was increased by the addition of fat to the ration, the nitrogen content of the milk was generally reduced.

At the instigation of the Association of Agricultural Experiment Stations in Germany,[180] and under the authority of the German government, ten agricultural institutions conducted experiments to determine the influence of rations rich and poor in fat upon the milk yield and composition of milk. Twenty cows were used at each station. The rations compared contained the same amounts of digestible matter, but one was richer in carbohydrate, and the other was richer in fat. The digestible fat in the one ration was 0.4 to 0.5 gram, and in the other, 0.9 to 1.0 gram per kilogram of body weight. The results indicated that this substitution of fat for carbohydrate was of no advantage. In fact, as a rule, it had a slightly unfavorable effect on both the yield of milk and of fat. The results of this cooperative investigation appear to conflict somewhat with those of Morgen and his collaborators, and to indicate that, if dietary fat has any favorable influence at all on the secretion of milk and fat in cows, it exerts this influence at a lower level of fat feeding than in the case of sheep and goats.

Some further light is thrown on the subject by the work of Jordan and his collaborators.[70, 71] The main object of this work was to show that cows can derive milk fat from dietary carbohydrate, and the evidence

adduced in favor of this view is very convincing. But some of the experimental results, which are not emphasized by the authors in drawing their conclusions, indicate that reduction of the fat in a cow's ration to a very low point interferes with her ability to secrete milk and milk fat. For instance, one of the cows used in the investigation was first fed for two weeks on a ration which contained 288 grams of fat daily. She was then changed to a ration which contained only 57 grams of fat and kept on low fat rations for the next ten weeks. During the period on the low fat rations her milk yield fell off rather rapidly. The percentage of milk fat was distinctly lower in the two weeks immediately succeeding the change to the low fat ration than it was initially. It then returned to about the initial level, but, on account of the falling milk yield, the total yield of fat was, of course, decidedly lower all through the period of low fat feeding than it was initially. The results suggested that cows respond to severe reductions in dietary fat by first reducing both the total yield of milk and the percentage of fat, and later still further reducing the milk yield without changing the total fat yield until the percentage of fat returns to the original level.

Jordan, Hart, and Patten [72] have carried out experiments on cows in which the phosphorus content of the ration was varied, while the yield and composition of the milk was studied. The phosphorus concentration in the milk was not affected, but the percentage of milk fat was noticeably reduced on the low phosphorus ration, and vice versa. The milk yield was little changed by the change in the food phosphorus; it tended to be a little higher on the low than on the high phosphorus ration.

The experiments of Cary and Meigs [23] have shown that food protein also has an influence on milk fat in the sense that reductions in the food protein often result in considerable reductions in the concentration of milk fat. The concentration of milk fat is decidedly more variable than that of either protein or lactose. In the experiments of Cary and Meigs the variation in the concentration of fat was 100/139 in the milk of individual cows.

Influence of food on the composition of milk fat. A great many experiments have been carried out to determine the effects of various kinds of feeds, not only on the composition of butter, but also on that of the body fat of swine. The results are much easier to understand in relation with what is generally known of fat metabolism; and, although this subject has already been discussed, it will be worth while to recall some of its main outlines.

Milk fat and body fat may be manufactured from either fat, carbohydrate, or protein in the food. When they are made from protein or carbohydrate they undergo numerous chemical changes within the body, and it is not to be expected that their composition could be predicted except from empirical studies of the kind of milk fat or body fat produced by an animal whose food is largely protein or carbohydrate. When they are made from food fat, on the other hand, it has been found that there is a very noticeable relation between their character and that of the fat

from which they came, and that the relation is, in a general way, such as would be expected from what is known of fat metabolism.

The food fats are broken down in the intestinal tract to glycerine and soaps of the fatty acids, and undergo several other chemical changes before they reach the mammary gland and the fat deposits within the body. It is not to be expected, therefore, that the triglycerides of the food should pass unchanged into the milk or body fat. But there is no known reason why fatty acids should not pass without further chemical breakdown from the intestinal tract through the blood to the mammary gland and other tissues, and there is very strong reason to believe that they do.

Ellis and Isbell [33] have made a detailed and illuminating study of the effects of food fat on body fat in swine. They find, not only that the iodine numbers and other characteristics of the body fat tend to be changed in the direction of those of the food fat, but also that certain individual fatty acids can be greatly increased in the body fat by feeding oils that are rich in them, and that certain other fatty acids, usually entirely absent from body fat, can be made to appear in it by supplying them in the food. This is, of course, not the same as saying that the body fat assumes the same composition as the food fat. There is always a certain amount of selection exercised in making body fat from food fat. Certain fatty acids pass easily from the latter to the former; others, with much more difficulty; and the body fat always tends to maintain some remnants of its usual character, even in the face of marked changes produced by peculiarities of the food.

That the situation regarding food fat and milk fat is in general similar to that regarding food fat and body fat is shown by the work of Morse, Eckles, Bowes, and others. Morse [101] and Eckles and Palmer [31] have shown that the feeding of various oils to cows raises the iodine number of their butterfat toward that which obtains in the oils; and Bowes [17] finds that arachidic acid is not usually present in the butterfat of goats, but can be made to appear there by feeding it as a constituent of peanut oil.

Butterfat, however, has certain peculiarities which complicate the problem of the changes produced in it by changes in the food. It contains a number of volatile fatty acids which are not found in the body fat of mammals or in the plant fats which commonly occur in the food of farm animals. The amount of these volatile fatty acids present in the butter has an effect on its melting point, and the influence of food on them is rather unpredictable.

The variations in the characteristic chemical constants of the milk fat produced when corn oil, linseed oil, and cottonseed oil are fed have been shown by Hunziker, Mills, and Spitzer [69] in Figure 41.

These results indicate that the iodine number increases in proportion to the numerical iodine value of the oil or fat fed, while the amount of volatile acids (R.M.No.) and the melting point remain fairly constant. Feeds rich in carbohydrate material have a tendency to increase the proportion of volatile acids.

The effect of various types of feeds upon the character of the milk fat has been well summarized by Hunziker, Mills, and Spitzer as follows: "Feeds rich in vegetable oils such as germ oil, corn oil, cottonseed oil, linseed oil, linseed meal, soy-bean oil, soy-bean meal, gluten feeds rich in fat, when fed in large quantities, also blue grass pasture, tend to increase the per cent of olein and decrease the per cent of volatile acids. This may

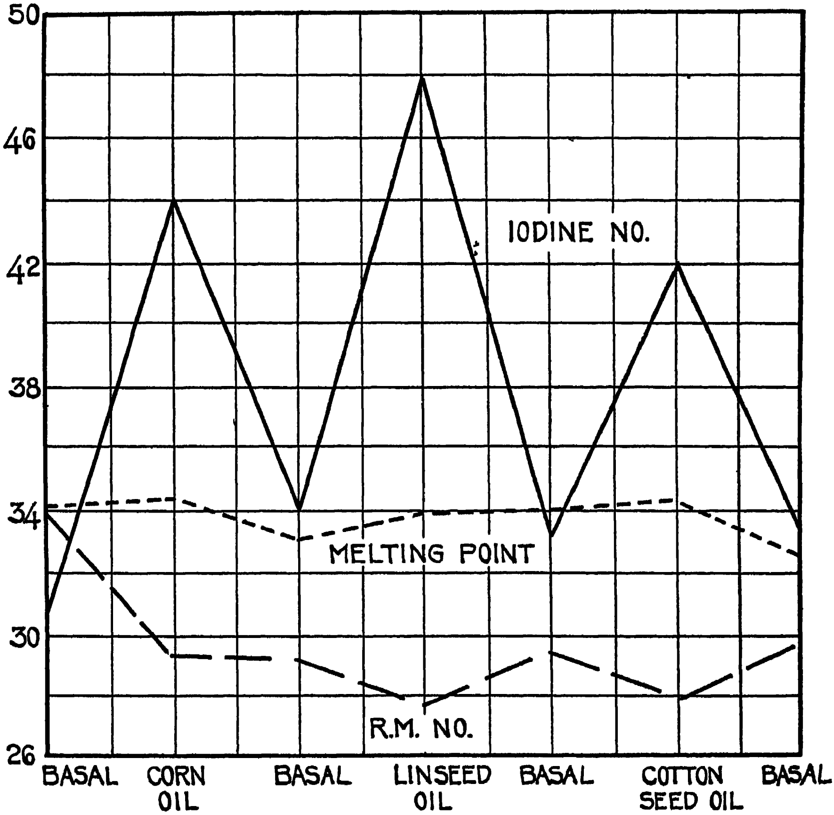

FIG. 41.—Effect of oil feeds upon butterfat constants.

or may not affect the melting point. With the exception of gluten feeds and pasture grass they tend to raise the melting point of the butterfat. Gluten feeds rich in fat and pasture grass lower the melting point.

"Feeds rich in carbohydrates and sugars, such as beets, mangels, beet tops, molasses, sweet corn fodder, corn silage, corn fodder, corn meal, buckwheat middlings, bran, etc., tend to increase the percentage of volatile acids and decrease the olein content. Their effect on the melting point is variable and not pronounced."

In addition to the character of the feeds, the distribution of the acids is determined to a lesser degree by climate, breed and care of the animals,

idiosyncrasy of individual animals, period of lactation, etc. The progressive decrease in the amount of volatile acids during the progress of the lactation period has been shown by Dons,[28] Paal and Amberger,[104] and others.

Milk secretion as related to mineral metabolism. This side of the subject of milk secretion has been studied rather unevenly. A great deal of attention has been devoted in recent years to the study of calcium and phosphorus metabolism in relation to milk secretion, while the experiments on the other aspects of the subject have been comparatively few and scattering.

The work on calcium and phosphorus has shown that the bones serve as a great storehouse for these elements, which can store up a surplus during periods of plenty and yield it up again in periods of need.[38, 39, 40, 41, 58] If either calcium or phosphorus is deficient in the food of a milking animal, she will take both elements from her bones and use which ever one is necessary to keep up the normal composition of the milk. The experiments of Hart and others [58] and of Meigs and others [95] indicate that this process may go on for three or four months with cows and may result in the loss of at least 20 per cent of the calcium and phosphorus contained in the animals' bodies. The milk yield is sometimes surprisingly well maintained in the face of the continued loss of calcium and phosphorus, though it usually falls off faster than it does when the dietary supply of these elements is adequate.[92]

A great deal of work has been done which bears on the question of the ultimate effects on milking animals of long-continued dietary deficiencies in either calcium or phosphorus. In a number of experiments, cattle which were fed for long periods on diets low in calcium showed various abnormalities in reproduction. They either failed entirely to breed, or gave birth to dead or weak calves somewhat before the end of the normal gestation period.[59, 60, 62, 92] A study of the details of these experiments shows, however, that the roughage in the low calcium rations consisted chiefly, or entirely, of various kinds of straw and of timothy hay. Recent work has shown that such rations are deficient in vitamin A as well as in calcium, and that the reproductive failures which have been observed are to be attributed to vitamin A deficiency rather than to calcium deficiency.[96]

In experiments recently carried out by the United States Department of Agriculture, and yet unpublished, cows have been fed for long periods on rations containing different quantities of calcium, while the calcium intake in the food and its output in the milk and calves were determined. At the ends of the periods, the animals were slaughtered and studies were made of the composition of their bones and, in some cases, of their whole bodies. The results show that, after periods of a year or more on rations containing about 25 grams of calcium daily, cows may readily utilize about 50 per cent of their calcium intake for the production of milk and calves. On such rations, however, they are likely to lose from 10 to 20 per cent of the calcium and phosphorus contained in their bodies. In the low calcium rations used in these experiments, vitamin A was prob-

ably also somewhat deficient, but some of the results suggest that, even when the vitamin A content of rations is sufficient, a calcium content of 25 grams daily or less is somewhat inadequate for Jersey cows which are capable of giving as much as 3,000 kilograms of milk annually.

Several investigators have studied the effects of adding bone meal and salts of calcium and phosphorus to rations low in their natural calcium content.[3, 4, 62, 81, 92, 106] In some of these experiments the results have been negative, but in others the addition of the calcium salts appears to have had rather small beneficial effects. A detailed study of these experiments indicates that the basal rations were deficient in vitamin A as well as in calcium and that this situation has somewhat clouded the results.

Studies of the conditions under which cattle are likely to suffer from phosphorus deficiencies have yielded much more definite and satisfactory results than the studies of calcium deficiency outlined above. This is partly because changes in the phosphorus content of the rations have immediate and marked effects on the phosphorus concentration of the blood,[90] and partly because rations which are low in phosphorus, but not in any of the vitamins, are of common occurrence. It appears that in many parts of the world the soils are low in their natural phosphorus content. When cattle are fed on the forage grown on such soil, and without any other liberal supply of phosphorus in their rations, they develop a characteristic pathological condition. Their joints become stiff, and they show an abnormal tendency to chew bones and other substances not usually eaten by cattle. Their milk yield is likely to be low, it is difficult to get them to breed, and the concentration of inorganic phosphorus in their blood plasma becomes very low. When they die or are slaughtered, their bones are found to be low in calcium and phosphorus content. All of these symptoms are relieved by supplementing their diets with bone meal or some other phosphorus source. An excellent review of the work in this field, with references to the literature, is given in an article by Eckles, Gullickson and Palmer.[32]

Feeding diets deficient in calcium and phosphorus has a very small and variable effect on the percentage of calcium and phosphorus in the milk. Fingerling[37] found that the concentrations of calcium and phosphorus in goat's milk were slightly increased after the animals had been for several weeks on a diet deficient in these elements. The figures obtained by Meigs and others,[95] on the other hand, would indicate that changing the quantity of calcium in the diet of cows has no consistent effect on the concentration of either calcium or phosphorus in the milk. In this work the variation of the calcium concentration in the milk was 100/131; that of the phosphorus concentration, 100/121.

A number of other investigators have studied the calcium and phosphorus content of milk, either while changes were made in the calcium and phosphorus of the diet,[79] or while NH_4Cl or $NaHCO_3$ were added to the diet.[88] The results were negative. Von Meysenbug[129] found that although the inorganic phosphorus in the blood serum of rachitic mothers was reduced, the phosphorus content of their milk was normal. Telfer[120]

obtained similar results as regards the Ca, P, and ash of milk. The work of Jordan, Hart, and Patten,[72] indicating that changing the dietary phosphorus of milking cows affects the fat, but not the phosphorus of their milk, has already been mentioned.

Only a small amount of work has been done on the changes produced by diet in the concentration of the soluble salts of milk. Denis and Sisson [26] noted considerable variations in the chloride content of human milk; but, as far as they were able to determine from observations on human subjects, the chloride content of the milk was not affected by the amount of sodium chloride ingested. They found that a dose of even as much as 500 grams of sodium chloride administered to a cow produced no appreciable change in the chloride content of her milk, and quote other investigators whose results show that moderate changes in dietary sodium chloride produce no effect on the composition of milk. Denis and Sisson did find, however, that, when very large doses of sodium chloride were given to a goat, so that the concentration of this salt in its blood was increased 18 per cent, and the milk yield was much reduced, there was an appreciable increase in the chloride content of the milk.

Before leaving the consideration of the work on the effects of dietary changes on such inorganic constituents of milk as chlorine and sodium, the interesting work of Van der Laan [125, 126, 127] should be mentioned. It will be found more fully discussed in the section on the effects of disease on the composition of milk. Van der Laan found that the osmotic pressure of milk remained the same as that of the blood of the animal from which it was taken, and, therefore, very nearly constant. As the osmotic pressure of milk is almost entirely due to its lactose and such soluble salts as sodium chloride, this means that there is a reciprocal relation between the concentrations of lactose and of the soluble salts in milk, the lactose increasing when the soluble salts are caused to decrease, and vice versa.

Several of the inorganic substances present in milk in very small quantities have been discussed in Chapter I. As was pointed out in Chapter XIV, several of these have been shown to be of great nutritive importance. The quantities in milk are often insufficient to provide for the requirements of animals fed milk exclusively throughout their lives, and the question has been raised as to whether the concentrations of such elements can be increased in the milk by appropriate feeding of the milking animal.

Some of the older investigations on iron appeared to indicate that the iron content of milk can be increased by adding iron compounds to the food of the milking animal; but, more recently, Elvehjem, Herrin and Hart [86] find by carefully planned experiments that this is not true. In a previous article they had pointed out that the older methods of iron determination are not satisfactory when applied to milk.[85]

Work on the feeding of iodine compounds to cows to increase the quantity of iodine in their milk has been reviewed by Maynard.[80] He gives figures showing that the iodine of milk has been increased in this way from 0.009 to 0.310 parts per million in one case, and from 0.063 to 0.720 parts per million in another. Several investigators report, how-

ever, that the feeding of iodine tends to lower the percentage and yield of milk fat.

The effects of changes in diet on the vitamin content of milk. Since the vitamins play such an important part in nutrition, it might be expected that there would be a strong tendency for their concentration in milk to remain practically constant like that of the protein, fat, carbohydrate, and most of the inorganic constituents. The study of this field is still very incomplete, but there is already plenty of evidence that milk varies widely in its content of some of the vitamins, and that the quantity of each vitamin in milk of most species of animals is largely influenced by the supply in the food.

Vitamin A. The facts pertinent to this discussion brought out in the accounts of vitamin A in Chapters IV and XIV may be summarized as follows. Vitamin A as found in cod-liver oil is an alcohol, the presence of which has so far not been demonstrated in plant materials and which probably does not exist there as such. Animals derive their supply of vitamin A from plants in the form of the yellow pigment, carotene. This pigment is converted wholly by some animals, and partly by others, into the alcoholic form of vitamin A, and appears in butter, sometimes wholly, sometimes partly, so converted. The butter of the goat, for instance, contains only the colorless form of vitamin A, but that of the cow often contains about equal quantities of the two compounds. The vitamin A of milk, whether in the form of carotene or colorless A, occurs practically exclusively in the fat.

That carotene passes readily from dairy feeds to cow's milk, and that the natural yellow color of cow's butter is dependent on the quantity of carotene in the feed, are facts that were shown a number of years ago.[105] More recently it has been demonstrated that the colorless vitamin A of cod-liver oil also passes readily into cow's milk.[48] Still more recently, three important investigations have been carried out in which the relationship between the quantity of vitamin A in cow's butter and that in the feed has been studied both by feeding experiments with rats [43] and spectrophotometrically.[11, 47] The three investigations are in good general agreement on many important points. In all three cases, plant products only were fed to the cows, and therefore the variations in the A content of the butter were brought about by variations in the carotene content of the feed.

Fraps and Treichler [43] found 50 Sherman units per gram of butter from cows on pasture, and only 2.5 units per gram of butter from cows on feed low in carotene. Baumann and Steenbock [11] found 27 gamma of carotene plus colorless A per gram of butter from cows on Wisconsin farms in July and August, and 11 gamma per gram in March and April. Gillam et al.[47] found about 12 gamma of carotene plus colorless A per gram of butter from cows on rations high in carotene, and about 3 gamma per gram of butter from cows on rations low in carotene. It is evident, therefore, that the A content of butter can be increased from two to twenty fold by increasing the carotene content in the feed of the cow.

The spread was greatest in the work of Fraps and Treichler, because their cows on the low carotene ration had no hay at all, while in the other investigations the cows were fed hay at all times. Work in the Bureau of Dairy Industry [96] has shown that hay is the chief source of vitamin A for dairy cows under ordinary winter feeding conditions, and that different kinds of hay differ greatly in their carotene content.

The three investigations all showed that the total A activity of the butter is reduced when the carotene content of the cow's feed is reduced. Those of Baumann and Steenbock, and of Gillam et al., both show that this reduction in the total A activity is caused by a large reduction in the carotene content of the butter and by a somewhat smaller, though still large, reduction in the colorless A content. The change in total A activity of the butter is, therefore, not mathematically proportional to the change in yellow pigment content, but there is, nevertheless, a strong tendency for the quantity of pigment and the quantity of total A activity to vary in the same direction.

As has been pointed out in Chapter IV, this strong positive correlation between the natural yellow color and the total A activity of butter is not found when butters from different species of mammals or of different breeds of cows are compared with one another. Certain breeds of cows have an hereditary tendency to secrete milk fat with a lower carotene content and a higher colorless A content than others, and certain mammals, such as the goat, secrete fat with the usual total A activity, but with no carotene at all. However, it has been shown by Palmer and others [105] and confirmed by unpublished recent work in the Bureau of Dairy Industry, United States Department of Agriculture, that the differences in the carotene content of the butters from different breeds of cows on the same feed are small in comparison to those which can be produced in the butter from individual cows by changing the carotene content of the feed. In general, therefore, the natural yellow color of cow's butter is an excellent approximate indication of its total A activity, and should be of great service practically in this respect.

In unpublished experiments of the Bureau of Dairy Industry, cows have been fed on rations containing pasture and different kinds of hay, and the effects of these rations both on the yellow color of the butter and on the nutritive properties of the milk have been studied. It has been found, in harmony with Palmer's conclusions, that the yellow color of the butter runs approximately parallel to the carotene content of the feed. The results have shown, also, that the vitamin A content of the milk as indicated by its tendency to promote the growth and survival of calves and rats, runs approximately parallel to the carotene content of the cow's feed, and, therefore, to the yellow color of the butter. A brief account of the work with calves has already been published.[96]

It may be seen from the lists of carotene-rich and carotene-poor feeds on page 108 that the only common dairy feeds rich in carotene are carrots, fresh green plant materials such as are found in pastures, and hay made from these plant materials in such a way as to retain the green color.

Work in progress in the Bureau of Dairy Industry has shown that, unless special precautions are taken, a large proportion of the carotene of fresh green plant materials is lost when they are dried, as in the process of making hay. This loss of carotene is approximately parallel to the loss in green color, and, therefore, the United States standard hay grades,[124] which are based largely on color, give an approximate indication of the carotene content of hay. Hay which is dried by certain artificial processes is usually greener and has a higher carotene and vitamin A content than the ordinary sun-dried hay.[110, 111] Table CXVI gives values obtained for the carotene content of a few dairy feeds. The determinations are not yet sufficiently numerous to give reliable averages, but the figures give as good a picture of the situation as possible at present. In addition to the facts already discussed, it shows that yellow corn, which is generally supposed to have a considerable vitamin A content, is in the same class as hay of the poorest quality. It is probable that most of the other grains and concentrates have carotene contents still lower than that of yellow corn.

Table CXVI.—Carotene content of some dairy feeds.

Feed	Carotene per gram of dry matter			Water	Number of determinations
	High	Low	Av.		
	Gamma	Gamma	Gamma	Per cent	
Fresh green alfalfa	412.0	267.0	326.0	79.6	5
U. S. No. 1 alfalfa hay	117.1	33.6	60.6	8.6	6
U. S. No. 2 alfalfa hay	16.3	13.7	15.0	8.6	2
U. S. No. 3 alfalfa hay	12.4	1.0	6.7	8.6	2
U. S. No. 1 timothy hay	24.5	9.0	18.9	11.6	3
U. S. No. 3 timothy hay	10.7	1.6	6.1	11.6	2
Carrots, yellow, garden	1128.2	709.4	948.7	88.3	4
Corn,* yellow, dent and flint	10.1	3.0	5.7	11.3	6

* The determinations with corn may include cryptoxanthine as well as carotene.

A study of Table CXVI shows that the vitamin A content of milk is likely to be highest when the cows are out on good pasture. Carrots are not very largely used as a dairy feed at present; and the A content of winter milks is likely to be lower than that of summer milks and to depend on the quality of the hay fed. It must be emphasized, however, that the carotene contents of both hay and pasture are subject to great variations. Recent work of Hart and Guilbert [64] has shown that cattle may suffer greatly from vitamin A deficiency when they are out on the range in dry seasons.

Vitamins other than A. The variations in the concentrations of these compounds in milk have been discussed in Chapter XIV and require only brief summary here. The vitamin D content of milk may be increased to about ten times its usual value by feeding vitamin D concentrates to the milking animal. Feeding large quantities of cod-liver oil to cows reduces the fat content of their milk.[63] Feeding large quantities

of vitamin D as irradiated ergosterol or irradiated yeast, however, does not have this effect, and no other objectionable effects have been noted in the studies made up to this time.[78, 117]

It has been shown that the quantities of vitamins C and B_2 in milk may be increased by appropriate feeding, but the largest increases observed so far have been to only about double the quantity originally present. The evidence indicates that the variations in the content of vitamin B_1 in milk that may be brought about by changes in the quantity of this vitamin in the diet is different for different species of animals. The fact that infants have sometimes developed beri-beri when they have been nursed by mothers whose diets were deficient in B_1, suggests that the concentration of this substance in human milk may be altered by choice of food. On account of the differences between the human and bovine intestinal tracts and the probability that B_1 may be synthesized by bacterial action in the bovine but not in the human intestine, it is quite possible that the B_1 content of human milk may be much more easily reduced by a deficiency of this vitamin in the diet than that of cow's milk.

There is no evidence as yet as to the variability of vitamin E in milk.

Transmission of drugs and poisons to milk. A number of statements occur in the older literature which would lead to the belief that drugs and poisons given to milking animals are largely excreted in the milk, so that the young of nursing mothers can be treated therapeutically through medicines given to their mothers, and are likely to be poisoned if the mother consumes any toxic substance. An example of the older views on the subject is to be found in an article by Baum.[10] More careful recent work, on the other hand, has shown that there is little or no tendency for most drugs and poisons to be excreted in the milk, although there are certain exceptional and rather startling cases which can not be overlooked.

Bucura [21] examined the milk of women who had received therapeutic doses of a great variety of drugs, the list comprising opium and some of its alkaloids, chloroform, alcohol, ether, quinine, antipyrin, aspirin, phenacetin, phenolphthalein, santonin, urotropin, stypticin, rhubarb, senna, cascara sagrada, colargol, salol, hydrastis, digitalis, and salts of tartaric acid, salicylic acid, iodine, bromine, lithium, mercury, iron, bismuth, and arsenic. Of these, the only ones found in the milk at all were aspirin, calomel, arsenic, iodides, bromides, and probably urotropin and even they were present only in very small quantities.

Other investigators have experimented with animals, administering large quantities of various drugs and compounds, and then examining the milk. Klingermann,[75] for instance, working with goats found no trace of alcohol in their milk after administering 25 or 50 cc. One hundred cc. given in the evening led to 0.15 to 0.30 of it in the milk the following morning; and even doses as large as 200 cc. in the evening led to only 0.35 cc. in the milk the next morning, although the milk yield was thereby reduced to one-third normal. Rosemann [108] obtained practically similar results with cows. Van Itallie [128] also worked with cows, and found that

physostigmin, pilocarpin, and morphine were not transmitted to the milk after subcutaneous injection, and that opium, sodium salicylate, salol, and turpentine oil were not transmitted after they had been given by mouth. Van Itallie studied the milk and urine of cows after they were given doses of fluorescein, phenolphthalein, rhubarb, and arsenic. All of these substances appeared in large quantities in the urine, while two of them, arsenic and fluorescein, appeared only in traces in the milk; and the other two not at all.

The investigations which have just been cited are sufficient to show that, as a rule, drugs and poisons are excreted only to a small extent or not at all in milk, but there is definite evidence to show that there are two plants in the United States, the poisonous principles of which form an exception to this rule. An account of this subject is to be found in articles by Wolf, Curtis and Kaupp [131] and by Marsh, Roe, and Clawson.[85] The plants in question are the white snake root (*Eupatorium urticaefolium*) and rayless goldenrod (*Aplopappus heterophyllus*). These plants are not relished by farm animals and are not eaten by them when plenty of other food is available. But large quantities are sometimes eaten when other food is scarce, and the animals are then likely to develop a disease called "trembles," which has a high mortality. Numerous cases of a disease with similar symptoms in human beings have been reported in regions in which trembles is common among the milch cattle, and experimental work has shown that the disease results from drinking the milk of animals which have eaten large quantities of either of the two plants above mentioned.

Effect of feed on the flavor and odor of milk. This subject has recently been extensively investigated by Gamble and Kelly [45] and by Babcock.[8] A brief summary of their work is to be found in the Yearbook of the United States Department of Agriculture for 1926. Much of what follows has been taken from the work of these investigators.

They point out that unusual flavors and odors may occur in milk from other causes than the feed consumed by the milking animal. It is well known, for instance, that chemical changes produced by bacteria or other causes may occur in milk after it has been taken from the cow and render it unfit to drink; and there is some reason to think that odors may sometimes be absorbed by milk directly from the surrounding atmosphere. These questions, however, can receive only passing consideration in this place.

Dombrowsky [27] found that the odors of iodoform, anise, phenol, turpentine, formaldehyde, and other materials were readily absorbed by milk. Finally, Ritland [107] came to the conclusion that the peculiar flavor so commonly noted in milk when turnips are fed to the cows came into it entirely through the air during milking and afterward.

Dean,[25] however, has shown conclusively that the flavor of turnips is transmitted to milk through the intestinal tract and blood of the cow, and his work has been quite fully upheld by the later results of Gamble and Kelly and of Babcock, above referred to.

These authors believe that, while feed-tainted barn air may have some effect on the flavor and odor of milk, it is of relatively small importance even under extreme conditions. They spread 150 pounds of corn silage fresh from the silo underneath two cows in a stable with an air space of only 500 cubic feet, with the doors and windows tightly closed. When milking was started one hour later, a strong odor of silage permeated the air, yet only rarely were the judges able to detect any more flavor or odor of silage in the milk from these cows than in that from two others milked in the open air.

In further confirmation of the view that the flavors of food pass to milk through the body of the cow, Babcock was able to detect the odor of garlic in samples of blood drawn from a cow 30 minutes after she had been fed two pounds of garlic tops. The blood gave an even stronger odor 45 minutes after feeding.

Having satisfied themselves that the flavors and odors of food pass to milk chiefly through the body of the milking animal, Kelly and his collaborators proceeded to investigate the question of what kinds of foods are likely to impart unusual flavors to milk, and the time relations between the period of feeding and the appearance of the change in the milk. They showed that, when corn silage, legume silage (alfalfa, sweet clover, or soy-bean), green alfalfa, cabbage, turnips, rape, or kale are fed to cows one hour before milking, they seriously affect the flavor and odor of the milk produced. The flavor of garlic may be detected in milk if the sample is taken from the cow one minute after she has been fed a half pound of garlic tops, but it increases with the passage of more time, reaches a high degree of intensity ten minutes after feeding, and persists for more than four hours. The odor of garlic is so strong and penetrating that it can readily be detected in milk a few minutes after the cows have inhaled it, even though they are not given any garlic to eat, and there is no garlic odor in the stable at the time of milking.

In contrast to garlic and other strongly-flavored foods, green rye, green cowpeas, Irish potatoes, dried beet pulp, and carrots affect the milk to only a slight extent when fed an hour before milking; green corn, green oats and peas, green soy-beans, pumpkins, and sugar beets have practically no effect at all.

Kelly and his collaborators also studied the length of time which it takes for the flavors of various foods to disappear from milk. They found that highly flavored foods may be fed immediately after one milking without seriously affecting the milk of the next one. The food flavors usually begin to decrease within four hours after feeding, and disappear within seven hours. This is true for even so strong a material as garlic, when it is consumed in small amounts, but large amounts of such highly-flavored foods as cabbage may produce a change which is detectable even in milk drawn twelve hours after feeding.

Most feed flavors are stronger in cream than in milk from which the cream was skimmed.

Ayers and Johnson [7] found that the garlic flavor may be removed from

milk by aerating at 63° C. (145° F.) for 30 to 60 minutes; and Gray and Eaton [54] applied this method on a large scale by blowing heated air through the milk. Gamble and Kelly, and Babcock find that, in general, aeration reduces strong feed flavors in milk, and that slight taints may thus be eliminated. If proper aeration, they say, follows the practice of feeding immediately after milking, most highly flavored feeds may be used without affecting the flavor and odor of milk.

Action of drugs, animal extracts, etc., on milk secretion. A very large number of investigations have been carried out to discover substances which, in addition to those in the ordinary diet, may be used to increase milk production. These investigations received much of their impetus from the discoveries (1) that the development of the mammary gland is brought about by some hormone mechanism, (2) that the initiation of milk secretion may occur independent of the nervous system, (3) that pituitrin injected into lactating animals produces immediate increase in the amount of milk that may be obtained from the udder, and (4) from the early observations that such condiments as fenugreek, fennel, sugar, anise, hay extract, etc., may be of use where poor and probably unpalatable feeding materials are used. Preparations of various sorts from almost every organ of the body have been tried—placenta, ovary, ovary without corpus luteum, corpus luteum, liver, pituitary, pineal body, thymus, fetal extracts, mammary glands, uterus, spleen, pancreas, adrenal bodies, kidney, intestines, thyroid, testicle, parotid, stomach, lungs, vagina, brain, heart, etc. Many drugs and other substances were also tried, such as pilocarpine, atropine, chloral, strychnine, digitalin, caffeine, potassium bromide, eserine, nicotine, muscarine, antipyrine, phloridzin, yohimbine, aloes, calomel, alcohol, castor oil, physostigmine, ginger, sulfur, antimony sulfide, fennel, anise oil, fenugreek, etc. Some of these substances have been administered subcutaneously, intravenously and by mouth to animals that in some cases were anesthetized and in some cases not.

Many of the references to the early work along this line are cited by Ott and Scott,[102, 103] who first investigated the action of extracts of the posterior lobe of the pituitary body.

These experiments in general have been carried out with one of two objects in view—(1) To throw light on the physiology of milk secretion; (2) To determine the practical usefulness of these substances for the purpose of increasing milk production. To the former investigations is due much of what is known today regarding the relation of milk secretion to the glands of internal secretion and the mechanism of control of this function (see above); but the latter, without exception, have yielded nothing of practical value, except to demonstrate clearly that no extracts, drugs, tonics, condiments, stomachics, stimulants, or powders or any special preparations of this sort can be relied upon to increase milk production. Many of the above substances were found to stimulate a pronounced immediate but temporary outpouring of milk; but none appears to lead to a decided increase in daily yield.

Of all of the above substances, pituitrin has yielded the most striking

effect, and has, therefore, been most thoroughly investigated. Ott and Scott administered it to a goat from one teat of which they were obtaining about 5 drops of milk during each 5-minute period. During the succeeding 4 minutes the yield was 405 drops, but during the next 5 minutes it fell back to 15 drops. A reinjection of the material led to a secretion of 75 drops. They published an experiment showing clearly that each successive injection of it is less and less effective. With an initial yield of 4 drops per 5-minute period, the first injection led to a yield of 101 drops, the second 32 drops, and the third 12 drops, the fourth 20 drops, the fifth 10 drops, and the sixth only 7 drops. These results were fully confirmed by Schäfer and Mackenzie,[118] but Gavin[46] showed with cows and Schäfer[114] noted with the human being that the daily yield of milk was not increased by the use of this substance no matter how administered. The work bearing on the mode of action of pituitrin has been reviewed by Rothlin, Plimmer and Husband.[109]

The futility of using various drugs, powders, stimulants, etc., in addition to a suitable and adequate diet, to increase milk production is most clearly brought out by the work of Simpson and Hill,[116] McCandlish,[83] Hays and Thomas,[65] and by Dyssegaard.[29] In general it may be said that where any of the above substances do stimulate milk secretion the effect lasts but a few minutes, there is frequently a subsequent compensatory decrease, and the yield over any considerable period of time is unaffected.

Pathological Conditions and Milk Secretion

Effects of pathological conditions on the composition of milk. Certain diseases and certain physiological changes to which cows are subject may produce profound changes in the composition of their milk. A great deal of effort has been expended in determining the nature and extent of these changes on account of the theoretical and practical interest of the subject. It has seemed likely from the theoretical side that a knowledge of the changes produced in milk by disease would throw some light on the nature of milk secretion. From the practical side, on the other hand, it is highly desirable to know whether any given abnormalities in the composition of milk indicate that the lactating animals are suffering from particular diseases, and also, if possible, to be able to distinguish between milk which is altered through some change, pathological or otherwise, in the condition of the cow and that which has been fraudulently adulterated. Unfortunately, much of the work on this subject has been done by investigators who were not primarily chemists; the tables given for the composition of milk often contain figures which are inconsistent with one another or have been rendered highly improbable by more careful work carried out later. In reviewing the subject, therefore, it is necessary to adopt a critical attitude and to give more attention to the rather few careful and systematic investigations than to the numerous hasty and uncritical series of analyses.

In studying the changes produced in milk by disease, it is necessary

to bear in mind that extensive changes in its composition may be brought about by certain physiological conditions of the lactating animal. The very marked differences between milk and the colostrum which is secreted for the first few days after parturition have been fully considered elsewhere. Another physiological state which is occasionally, though not usually, accompanied by considerable changes in the composition of milk is that of heat in the milking animal. Stern [118] has made a fairly systematic study of this subject, and gives a table showing the quantity, specific gravity, fat, and fat-free dry matter of the milk of a number of cows when in heat and when not in heat. His results show that the quantity of milk is usually a little reduced in the heat periods. The fat is likely to be a little increased, but is quite variable, and occasionally markedly decreased. The specific gravity and fat-free dry matter are only slightly altered, and just about as often in one direction as in the other.

Changes also take place in the composition of the milk when the cow is being dried off toward the end of her lactation period. Koestler and Elser [76] have discussed these changes and given tables showing their extent. The casein, albumin, fat, chlorides and other salts of the milk are moderately increased in concentration, while the lactose is somewhat decreased. If the udder is free from bacterial infection, these changes are very moderate in extent. But infections of the udder are more common than is generally supposed, and, when they exist, they are likely to accentuate greatly some of the changes above described and to introduce new ones which will be more fully considered later, so that the milk becomes unfit to drink.[77]

The diseases which affect the composition of milk group themselves naturally into those in which the udder is directly involved and those which affect primarily other parts of the body, leaving the mammary-gland cells in a more or less nearly normal condition. Amberger [2] has made a study of the composition of milk from inflamed udders, taking samples on a number of successive days during the course of the disease, and comparing in each case the milk from the affected quarter with that from the other normal or less affected quarters. Two cows were studied, in one of which the disease was severe and was followed by death, while the other animal had a mild case from which she recovered in the course of five days. In the severely affected animal the composition of the milk from the affected quarter approached that of the blood in many respects. The fat and sugar were reduced to less than one per cent while the nitrogen was higher than normal. The ash contained much more chlorine and less of other constituents than does normal milk. The results as a whole strongly suggest that the secreting cells of the affected quarters were so altered by the disease that they retained little power either of preventing the passage of the blood constituents into the milk ducts or of converting these constituents into those of milk.

Amberger's other results furnish a good example of the very variable manner in which the composition of milk may be affected by disease. The milk from the three sound, or comparatively sound, quarters of the

severely diseased cow was much more closely normal than that from the most affected quarter, but not entirely so. The lactose was rather low throughout, while the fat varied from about 2 per cent to over 9 per cent.

In the mildly diseased cow studied by Amberger the milk from the three sound quarters was normal, as far as can be judged from the figures given, while that from the affected quarter showed a comparatively slight alteration, but generally in the same direction as in the case of the severely diseased cow. The lactose was low throughout, the protein high, and the chlorine slightly high. The fat was very high at first, but soon became normal.

In addition to Amberger, Seel [115] has studied the milk from the affected and unaffected quarters of cows with mastitis while Storch,[119] Guillebeau and Hess,[55] and Zaribnicky [134] give analyses for the mixed milk from the affected and unaffected quarters of inflamed udders. The results on the whole are not inconsistent with those of Amberger. The general conclusions indicated by all of these investigations may be stated as follows. The composition of milk from inflamed udders, and particularly from the immediately affected quarters tends to approach that of the blood. The most usual change is a reduction in the lactose which may be very extreme if the disease is severe. The protein is commonly increased, while the fat is variable, but may be very much reduced in severe cases. The investigations of Seel and Zaribnicky bring out the interesting fact that the protein of the abnormal milk is likely to contain much more "albumin" and less casein than is usual—another indication that the cells of the diseased gland often permit the passage of the blood constituents into the milk ducts. Still another indication in the same direction is the high chloride content of the milk from inflamed udders.

From what has just been said it is clear that fairly marked and characteristic changes often appear in the milk from cows with diseased udders. But the results obtained in milk from cows with other kinds of disease have been very variable. Before taking them up it will be advantageous to consider certain work of Van der Laan which has established a physiological law applying to the secretion of milk under both normal and pathological conditions. Van der Laan [125, 126, 127] has made a careful study of the osmotic pressure as determined by the freezing point depression in the milk, blood, and bile of both normal and diseased cows. He finds that the freezing point depressions in the milk, blood and bile of any given animal are equal to each other whether the animal be normal or diseased. If the blood freezing point be raised or lowered by a general infection like anthrax, by the administration of drugs, or by water enemas, the freezing point of the milk promptly changes to correspond with it. But as the freezing point of the blood can be only very slightly altered without bringing about the death of the animal concerned, the freezing point of milk remains correspondingly constant under all sorts of pathological conditions and constitutes one of the best criteria that we have for judging whether any particular sample of milk owes its abnormal composition to something that has occurred within the body of the cow before it was

taken from the udder, or to some form of subsequent adulteration. The importance of this law for the subject of milk from diseased cows will be more fully seen when we come to the consideration of some of the later and more critical investigations of the subject.

In 1914 Zaribnicky [134] published an article on the effects of disease on the composition of milk. He gives a fairly extensive bibliography and a table showing his own analyses of the milk from cows with a variety of diseases, including a few cases of mastitis. The table contains a good many inconsistencies, which are not fully explained in the text, and which seem to indicate faulty analytical work, but it is interesting as being fairly typical of many of the results which have been obtained in this field. The high figures for the "albumin" in the milk of cows with mastitis are interesting while the great variation in the milk fat of cows with foot and mouth disease (from 2.2 per cent to 19.5 per cent) illustrates well the very varying effects produced on the composition of milk by different phases of the same disease.

Bergema [12] has made a comprehensive study of the composition of milk from cows with a considerable variety of diseases, and has published a very full account of his results. He concludes that milk is usually altered by digestive diseases, and often also by other diseases when these are severe enough to cause a marked reduction in the milk yield. He considers that some of the changes produced in milk by disease are sufficiently uniform to warrant the making of general statements. According to his results, for instance, the chlorides in the milk of diseased cows are usually increased; while the lactose, if it is changed at all, is likely to be diminished. The fat is, on the whole, more likely to be increased than diminished; and the total protein is changed about as often in one direction as the other, or may remain normal. The albumin, however, is likely to be increased.

Koestler and Elser [76] have made a very careful study of the effects of foot and mouth disease on the composition of milk. They used cows which had been under observation for many months before they acquired the disease. Not only had samples of milk from the separate quarters of the udders of these animals been analyzed under normal conditions but the bacteriology of the separate quarters had been studied. Further the leucocyte count of the milk was determined both before and after the onset of the disease, so that it was possible to judge whether any given animal was suffering only from the effects of the general infection or whether there was also accompanying inflammation of the udder.

One of the most noticeable effects of the onset of the disease, whether the udder was inflamed or not, was a very marked reduction in the volume of the milk secreted—often to one-quarter of its original quantity. The changes in the composition of this very much reduced milk yield depended on whether or not the udder was inflamed. If it was not inflamed, as shown by a low leucocyte count in the milk, the changes in composition were moderate. They consisted in an increased fat, protein, and ash content and in a decreased lactose content. The fat content was very vari-

able. It was usually under 10 per cent, but sometimes rose to between 10 and 15 per cent at one stage or another of the disease. The protein content was often increased to between 4 and 5 per cent, the normal for the cows used being between 3 and 3.5 per cent. The lactose was frequently reduced from a normal between 4.5 and 5.0 per cent to between 3.0 and 4.0 per cent, but rarely below 3.0 per cent. The ash was commonly only slightly increased.

More detailed studies of the abnormal milk brought out further interesting results. It was found, for instance, that the casein and "albumin" fractions of the milk protein were both increased, the latter perhaps usually somewhat more than the former. Among the ash constituents, the chlorides were quite constantly and markedly increased. The depression of the freezing point was normal or only slightly greater than normal.

In the case of milk which contained large numbers of leucocytes and came, therefore, from inflamed quarters of the udder, the changes were very much more marked than those which have just been described and similar to those which other investigators have recorded for milk from cases of mastitis. It is to be remembered that in cases of mastitis the casein and fat of milk are very markedly reduced instead of being increased; that the lactose is reduced to very low figures; and that the protein other than casein, and the chlorides, are very markedly increased.

Koestler and Elser give an illuminating discussion of their results. They point out that, when a cow is dried off toward the end of her lactation period, changes occur in the composition of the milk which are similar to the changes which they find in the milk from cases of foot and mouth disease uncomplicated by inflammation of the udder. The casein, fat, and chlorides are increased, while the lactose is moderately reduced. Drying a cow off involves usually a considerable reduction in her feed combined with less frequent milkings. Koestler and Elser suggest that there is a parallelism between these conditions and those which occur in severe disease; in the latter case also there is a marked decrease in the food intake, and the milkings are very apt to become less frequent. They put forward the hypothesis that the most noticeable changes in the composition of milk from cows affected by severe disease uncomplicated by udder inflammation are mainly the result of the sudden great decrease in milk yield which is brought about largely by decrease in the food supply combined with difficulty in milking regularly. In the light of this hypothesis they compare their own results with those obtained by previous investigators, and they believe that the previous results can on the whole be regarded as being in very fair agreement with their conclusions.

The work of Koestler and Elser has certainly added greatly to our knowledge of the effects of disease on the composition of milk, and seems to the writers to offer a satisfactory explanation of the agreements and apparent disagreements that are to be found in the work of previous investigators. The whole body of work seems definitely to justify the conclusion that the most constant and characteristic change produced in milk by disease is an increase in the chloride content accompanied by a

decrease in lactose and a nearly unchanged depression in the freezing point. Milk from diseased cows can, therefore, be distinguished from watered milk, when it is possible to make freezing point and chloride determinations. But it must always be remembered in this connection that cows may often be suffering from fairly severe disease without showing any noticeable change at all in the composition of their milk.

It has not been necessary to refer to many articles in the foregoing discussion, because the publications of Koestler [76] and Bergema [12] contain references to the most important previous work on the effect of disease on the composition of milk. Some recent work, however, is not referred to in their bibliographies. Hortvet [68] has reviewed exhaustively the question of the freezing point of milk and Tocher [121] gives extensive tables showing the variations in the composition of normal milk.

Summary Outline of the Physiology of Milk Secretion

The physiology of milk secretion is such a complicated subject in itself and so bound up with the problems of general physiology that it is very difficult to give any picture of it in the form of a short outline. Some of its important aspects, however, may be stated as follows.

In a general way, milk is manufactured within the body of the milking animal from the chemical compounds contained in her food. The earlier stages of the chemical changes which take place in the food, however, are just the same in milking as in non-milking animals; the proteins are converted to amino acids, and the carbohydrates to dextrose in preparation for milk manufacture just as in preparation for the building of body tissue or for use as fuel. These earlier stages of chemical change are carried out in the digestive tract, in the liver, and in other parts of the body exclusive of the mammary gland; the intermediate products resulting from them are thrown into the blood, are taken out of that fluid by the gland, and undergo within it the changes to casein, lactalbumin, milk fat, and lactose, which are characteristic of milk secretion.

The amino acids, blood fats, and dextrose, which result from the first changes undergone by the foods, are to be regarded as the general currency of metabolism and may be converted into muscle, body fat, and stores of body glycogen, instead of into milk constituents. Conversely, the body constituents above mentioned may be converted into general currency and from that into milk constituents during periods of food shortage. While the milk constituents come ultimately from the food, therefore, they do not necessarily do so in any immediate sense; and the milk yield can not be depended upon to give an immediate answer to the question whether or not a given ration is adequate. In order to have a complete picture of the relations between food supply and milk secretion at any given period, we must have information not only about the food and milk but also as to whether the animal's stores of body substance are increasing, stationary, or decreasing.

Animals are not compelled to make body or milk fat from food fat,

or body or milk carbohydrate from food carbohydrate. They may make either fat or carbohydrate from food protein, and fat from food carbohydrate.

In milking animals, the general result of any deficiency in the food supply is a falling off in milk yield at more than the normal rate, and, at the same time, a loss from the animal's body of material suitable to supply the deficiency. The extent to which dietary deficiencies affect milk yield depends on the nature and extent of the deficiency, on the state of the animal's body stores, on the stage of lactation, and on individual peculiarities of the animal. It is not surprising, therefore, that the experiments, in which the effects of dietary changes on milk yield have been studied, have had very variable results.

Milking animals have a strong tendency to secrete milk of nearly uniform composition in the face of large changes in their diet. The physiological basis for this situation is to some extent revealed in the account contained in the foregoing paragraphs. The food constituents are generally changed in the direction of taking a certain standard chemical form before entering the blood; the concentrations of amino acids and dextrose in the blood are regulated within narrow limits by unknown physiological mechanisms; and the mammary gland controls the relative amounts of these constituents which it takes up, even in the face of variations which may take place in the composition of the blood. But, in spite of all these regulatory arrangements, the composition of milk can be affected by changes in the diet. The milk fat is probably the constituent most easily affected. Its concentration may be moderately changed under appropriate conditions, and various fatty acids not normally present may be introduced into it when they are supplied in the food. Small changes in the concentrations of the other constituents of milk also can be induced, at least temporarily, by dietary changes.

In regard to the vitamins, which play so large a part in giving to milk its unique dietary properties, it is surprising to find that their concentration in milk may vary greatly. The concentrations of most of them, as far as is known, are largely dependent on the supply in the food of the milking animal; this influence may give rise to a ten or even twenty fold change in the concentration of vitamin A in milk.

Most foreign substances introduced into the blood either fail altogether to pass through the mammary gland cells into the milk, or pass to only a very small extent. But there are some important exceptions to this rule.

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